COMPARACIÓN BIOGEOGRÁFICA DE LAS POBLACIONES DE *QUERCUS ILEX* L. TEST DEL MODELO NÚCLEO-PERIFERIA

TESIS DOCTORAL

ANA GARCÍA NOGALES

UNIVERSIDAD PABLO DE OLAVIDE

DEPARTAMENTO DE SISTEMAS FÍSICOS, QUÍMICOS Y NATURALES

ÁREA DE ECOLOGÍA

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Memoria que la Licenciada Ana García Nogales presenta para optar al Grado de Doctora en Ciencias Ambientales por la Universidad Pablo de Olavide

Esta Memoria ha sido realizada bajo la dirección de: Dr. José Á. Merino Ortega, Dr. José I. Seco Gordillo y Dr. Juan C. Linares Calderón

Lic. Ana García Nogales

Aspirante al Grado de Doctora

Sevilla, diciembre de 2016

Dr. José Á. Merino Ortega, Catedrático de Ecología de la Universidad Pablo de Olavide (Sevilla)

Dr. José I. Seco Gordillo, Profesor de Ecología de la Universidad Pablo de Olavide (Sevilla)

Dr. Juan Carlos Linares Calderón, Profesor de Ecología de la Universidad Pablo de Olavide (Sevilla)

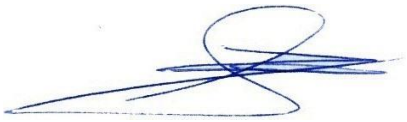
INFORMAN

Que esta Memoria fue realizada bajo nuestra dirección en el Departamento de Sistemas Físicos, químicos y Naturales de la Universidad Pablo de Olavide de Sevilla. Ante lo cual, considerando que representa el trabajo de la Tesis Doctoral, autorizamos su presentación en la Comisión de Doctorado.

Para que así conste, lo firmamos en Sevilla, a 5 de diciembre de 2016.

Dr. José Á. Merino Ortega

Dr. José I. Seco Gordillo



Dr. Juan C. Linares Calderón

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A mis padres, Loli y Antonio

A mis Leandros

0. RESUMEN

Las especies vegetales que presentan un amplio rango de distribución suelen exhibir una alta variabilidad intraespecífica como respuesta a la gran diversidad de combinaciones de valores de los factores ambientales a la que están sometidas a lo largo de su área de distribución. En el presente estudio se han llevado a cabo una serie de trabajos de campo, experimentos de invernadero y en condiciones controladas (cámaras de cultivo) con el fin de evaluar la variabilidad intraespecífica en la encina (*Quercus ilex* subsp. *ballota*), intentando dilucidar posibles patrones espaciales relacionados con la posición biogeográfica de las poblaciones consideradas. Para ello, se seleccionaron 12 estaciones de muestreo correspondientes a poblaciones de encina que abarcan el rango latitudinal de la especie (desde el sur de Francia hasta el sur de Marruecos); cubriendo así una gran variedad de hábitats con características climáticas contrastadas.

Los árboles de las poblaciones ubicadas en la periferia del gradiente latitudinal mostraron una altura, área basal, diámetro de copa y concentración de nitrógeno foliar significativamente menores que los árboles de las poblaciones del núcleo. Asimismo, los frutos de las poblaciones periféricas eran de un tamaño significativamente menor que los de las poblaciones centrales; todo ello sugiriendo un mayor grado de estrés en las poblaciones periféricas.

La caracterización fenotípica realizada sobre los caracteres morfológicos y fisiológicos de las plántulas creciendo en condiciones controladas, mostró que los individuos procedentes de la periferia del área de distribución poseían un aparato fotosintético menos eficiente que el del resto de poblaciones; resultado que se puede interpretar como una posible respuesta adaptativa a los hábitats más estresantes de la

periferia. Paralelamente, estos indiviuos de la periferia mostraron una relación Fracción de biomasa aérea/Fracción de biomasa subterránea significativamente mayor que la de las poblaciones del núcleo; lo que se puede interpretar como mecanismo de compensación a la baja eficiencia fotosintética de los primeros.

Además, se realizaron dos experimentos en los que se sometió a las plántulas de las 12 poblaciones dos situaciones distintas de estrés: por un lado, a fumigación con ozono en condiciones homogéneas de temperatura, humedad e intensidad luminosa constantes (cámaras de cultivo); y por otro, a los cambios estacionales de la temperatura ambiental (cultivos al aire libre). Las poblaciones nativas de la periferia mostraron la mayor tolerancia intrínseca al ozono, ya que manifestaron un mejor rendimiento fotoquímico en condiciones de fumigación en comparación a las poblaciones nativas del núcleo. En el caso de la respuesta a los cambios estacionales de temperatura, no se observó ningún patrón convergente entre las poblaciones periféricas; siendo las poblaciones procedentes de hábitats más fríos y con mayor oscilación térmica las que mostraron mayor plasticidad adaptativa tanto a las altas como a las bajas temperaturas.

Los resultados del presente estudio ponen de manifiesto la alta variabilidad intraespecífica de la encina, y que ésta recae en gran parte en las poblaciones que habitan los márgenes del área de distribución. El presente estudio viene a confirmar que las poblaciones periféricas tienen un importante valor para la conservación, ya que éstas pueden comportarse como depósitos de recursos genéticos, de plasticidad fenotípica y, por todo ello, de capacidad adaptativa. Por todo lo cual, es importante la incorporación de la variabilidad intraespecífica a los modelos de proyecciones futuras que estudian los posibles cambios de distribución geográficos de las especies como consecuencia del Cambio Climático.

I. INTRODUCCIÓN GENERAL

1. MARCO CONCEPTUAL

El análisis de la estructura del área de distribución de una especie es un tema clásico en los estudios de Ecología, sobre todo a partir de los trabajos de McArthur (1972) y Rappoport (1982). El enfoque del área de distribución como una dualidad núcleo-periferia ocupa actualmente un lugar prominente en los estudios de Ecología Evolutiva y en el campo de la Biología de la Conservación. El análisis de la estructura poblacional del área de distribución de una especie es necesario para conocer la heterogeneidad funcional de las especies, para hacer predicciones sobre las respuestas de éstas a los cambios ambientales (por ejemplo, al Cambio Climático) y para el desarrollo de estrategias de gestión y conservación.

Las especies vegetales longevas que presentan extensas áreas de distribución generalmente están sometidas a una gran variedad de condiciones bióticas y abióticas, tanto en el espacio como en el tiempo (Gaston, 2003). En principio, la existencia de poblaciones localizadas en áreas caracterizadas por condiciones ambientales contrastadas, combinada con una cierta limitación en el intercambio de información genética (Sexton *et al.*, 2009), suele resultar en la selección de individuos en armonía con las condiciones ambientales locales y, consecuentemente, en la existencia de poblaciones con características genéticas distintas (ecotipos o razas ecológicas) (Bruschi, 2010; Arend *et al.*, 2011). Paralelamente, las plantas pueden responder a la variabilidad ambiental mediante la plasticidad fenotípica de sus caracteres funcionales, es decir, mediante la capacidad que tiene un mismo genotipo a variar su fenotipo bajo diferentes condiciones ambientales (Pigliucci, 2006). De acuerdo con todo ello, la variación intraespecífica de los

caracteres en una especie representa la expresión directa tanto de su variación genética como de su plasticidad fenotípica dentro de una especie (Aubin *et al.*, 2016).

La capacidad adaptativa de los organismos se define como la habilidad de éstos para ajustarse a los cambios ambientales espaciales y temporales; y está determinada por la diversidad genética y por la plasticidad fenotípica de la especie (Aubin *et al*., 2016). Las respuestas plásticas parecen ser igual de importantes que la variabilidad genética en relación al potencial evolutivo de las especies en cuanto a su persistencia, especialmente cuando se trata de procesos adaptativos rápidos como es el caso de los hipotéticos cambios que proyectan los distintos escenarios de Cambio Climático (Hoffmann & Sgrò, 2011); lo que hace que el estudio de la capacidad adaptativa de las especies sea crítico para la gestión de los ecosistemas (Bertrand *et al.*, 2011; Alberto *et al.*, 2013; Aranda *et al.*, 2015).

Los análisis de vulnerabilidad en el contexto del Cambio Climático se suelen basar en modelos de nicho climático, los cuales sufren de fuertes limitaciones a la hora de predecir futuros cambios en la distribución de las especies (Wiens & Graham, 2005), ya que éstos no incluyen la diferenciación poblacional dentro de las especies, es decir, no incluyen su capacidad adaptativa (Davis & Shaw, 2001; Valladares *et al*. 2014). Las especies arbóreas pueden mitigar los efectos esperados del Cambio Climático, bien mediante el potencial de las poblaciones para responder, a través de procesos microevolutivos, en nuevas composiciones genotípicas; o bien mediante la migración de especies y poblaciones a nuevas áreas (Davis & Shaw, 2001); generando, en este último caso, cambios en el área de distribución (Savolainen *et al.*, 2007). Dado que la migración de los árboles es lenta en relación al ritmo del Cambio Climático, es importante que las predicciones sobre los efectos de este fenómeno tengan en cuenta los posibles cambios evolutivos de la especie (Savolainen *et al.*, 2007). Debido a la escasez de información

sobre las relaciones del potencial evolutivo, la adaptación y la persistencia de las especies en ambientes cambiantes; en las últimas dos décadas se ha puesto de manifiesto la necesidad de aumentar el conocimiento del potencial evolutivo y la capacidad de adaptación de las especies en un contexto de Cambio Climático (Matesanz *et al.*, 2010).

En principio, la plasticidad fenotípica tiende a maximizar el ajuste biológico en ambientes cambiantes (Ackerly *et al.*, 2000; Matías & Jump, 2015), y se sugiere que una mayor plasticidad fenotípica puede ser una propiedad común para las poblaciones nativas de ambientes estresados, como son aquéllas de los bordes del área de distribución (Volis *et al.*, 1998). Sin embargo, algunas evidencias experimentales demuestran que la diferenciación genética puede estar favorecida frente a la plasticidad en ciertos ambientes extremos, en los que el costo inherente de la plasticidad sobrepase los beneficios de mantenerla (Parsons, 1991; Meier & Leuschner, 2008; Mägi *et al.*, 2011).

La variabilidad en los fenotipos de una especie como consecuencia de la selección a lo largo de gradientes ambientales puede resultar en patrones espaciales biogeográficos en ciertos caracteres dentro de su área de distribución. El estudio de individuos nativos de distintos orígenes geográficos, pero cultivados en condiciones homogéneas, permitiría la detección de diferencias constitutivas en caracteres concretos dirigidos por una presión ambiental selectiva en el hábitat donde estas poblaciones han evolucionado (Ramírez-Valiente *et al.*, 2010). Por ejemplo, en una serie de trabajos sobre de *Quercus suber* se ha detectado que la resistencia a la sequía de las poblaciones disminuye con la latitud (Ramírez-Valiente *et al.*, 2009), en tanto que la resistencia a las bajas temperaturas parece aumentar con la latitud (Aranda *et al.*, 2005); sugiriendo la existencia de distintas adaptaciones a las condiciones ambientales locales en función de la procedencia de los individuos estudiados dentro de un gradiente ambiental.

2. EL MODELO NÚCLEO-PERIFERIA

A gran escala, sería de esperar que las características diferenciales de las poblaciones de una especie tuviesen una clara manifestación biogeográfica, ya que, al menos desde el punto de vista físico, los extremos del área de distribución suelen ser donde el nivel de estrés para la especie es más alto; en tanto que –en términos de promedio– en el núcleo del área de distribución dominarían las condiciones más próximas al óptimo fisiológico.

Así, el modelo clásico de área biogeográfica de una especie subraya su fuerte carácter heterogéneo, con marcadas diferencias entre las poblaciones que forman el núcleo del área de distribución y las poblaciones periféricas (Brown *et al*., 1996; Volis *et al.*, 1998; Hampe & Petit, 2005). Esencialmente, esta heterogeneidad es explicada en base al cambio en las condiciones ambientales dominantes desde el núcleo del área hacia la periferia. Así, los límites están caracterizados por la mayor importancia de los factores abióticos (sobre todo climáticos) frente a los bióticos, por los valores extremos de estos factores; así como por el carácter variable e inestable de la matriz ambiental (Sexton *et al.*, 2009); todo lo cual favorece además una situación de fuerte aislamiento reproductivo entre las poblaciones de la periferia. En este escenario, la selección lleva a genotipos que favorecen la supervivencia en condiciones adversas, es decir, a individuos caracterizados por su resistencia al estrés. Es por ello que las poblaciones periféricas pueden resultar muy importantes a la hora de diseñar planes de conservación de especies (Channel & Lomolino, 2000; Hampe & Petit 2005).

En los límites septentrionales de las áreas de distribución, se producen situaciones de estrés estacional debido a la baja temperatura del aire y a la congelación del agua en el suelo; lo que lleva, por una parte, a la limitación de sales (sulfatos, nitratos, fosfatos, etc)

y, por otra, a la limitación de CO_2 debido al cierre estomático. La combinación de una baja temperatura y de alta iluminación, propia de los ambientes mediterráneos, produce desacoples entre la fase luminosa y la fase oscura de la fotosíntesis (es decir, fotooxidación). El exceso relativo de electrones y la falta de aceptores (nitratos, sulfatos, dióxido de carbono) llevan al bloqueo de las rutas electrónicas y, consecuentemente, a la desviación de electrones de sus rutas metabólicas y a la formación de radicales libres (ROS) en el cloroplasto (García-Plazaola *et al.*, 1999); en tanto que la limitación de fosfatos puede llevar a desacoples en la cadena respiratoria con la formación de ROS en la mitocondria. Por su parte, las altas temperaturas en los límites más meridionales de las áreas de distribución de las especies mediterráneas, llevan a la desnaturalización de los enzimas y a la consiguiente alteración de las membranas celulares, lo que obliga a la síntesis de proteínas protectoras (Larcher, 2000). Además, la combinación de las altas intensidades luminosas y de la baja disponibilidad hídrica propias de esas localizaciones, provoca también desacoples en el cloroplasto como los ya comentados y la formación de radicales libres.

Evidentemente, el establecimiento de los límites del área de distribución resulta de un proceso muy complejo, que no sólo es producto de las interacciones con los factores climáticos y edáficos, sino también de las diversas interacciones bióticas que se establecen entre las especies presentes; como lo prueba el hecho de que los límites tienden a permanecer relativamente estáticos a pesar de las frecuentes variaciones de los valores de factores ambientales (Gaston, 2003; Hampe & Petit, 2005). Esta alta complejidad de interacciones y los valores extremos de algunos de los factores abióticos pueden haber llevado a las poblaciones de la periferia a la resistencia a una multiplicidad de factores de estrés (véase por ejemplo Hoffmann & Parsons, 1991).

Las plantas nativas de la Cuenca Mediterránea (y, en general en las plantas procedentes de climas de tipo mediterráneo) han evolucionado en un contexto caracterizado por valores extremos estacionales de diversos factores abióticos (sequía, alta temperatura, intensa radiación solar, suelos generalmente pobres y desequilibrados); todos ellos asociados a la generación de estrés oxidativo (ver Bussotti, 2008). Es por ello que estas especies deberían contar con amplias dotaciones defensivas fisiológicas y bioquímicas contra los radicales libres (García-Plazaola *et al.*, 1999), lo que, dado el carácter universal de estos mecanismos podría conferir a estas especies alta resistencia genérica al estrés oxidativo (resistencia cruzada) (García *et al.*, 1998; Paoletti, 2006; Bussotti, 2008). Por otra parte, las poblaciones muy estresadas, como son las de alta montaña, o las expuestas a contaminantes atmosféricos, tienden a presentar concentraciones de complejos defensivos y de reparación constitutivamente más altas que las poblaciones que soportan grados inferiores de estrés (Leipner *et al*, 1997, Laureano *et al*., 2016); lo que podría explicar, por ejemplo, la mayor resistencia a la fumigación con SO_2 observada en las plántulas de *Quercus ilex* L. nativas de ambientes más estresados cultivados en ambientes libres de estrés (cultivos controlados) (García *et al.*, 1998). En este sentido, cabría esperar que las poblaciones ubicadas en la periferia del área de distribución, al ser las más estresadas, sean las poblaciones genéricamente más resistentes al estrés.

3. ESPECIE DE ESTUDIO

La encina (*Quercus ilex* L.), perteneciente a la familia *Fagaceae*, es una especie perennifolia que presenta una distribución circunmediterránea (Figura 1); aunque es mucho más abundante en el Mediterráneo occidental. La encina es una especie relativamente

indiferente a la naturaleza del sustrato; encontrándose tanto en suelos margosos como pedregosos; tanto calizos como silíceos (González-Bernáldez *et al.,* 1977; Jiménez *et al.*, 1996). La media de precipitación en sus hábitats suele estar entre 600 mm y 300 mm, habitando en un rango temperaturas medias que oscilan entre -3ºC en enero y 28ºC en agosto (Jiménez *et al.,* 1996). Dentro de la especie existe un alto polimorfismo, a nivel interpoblacional, intrapoblacional e intraindividual. Un ejemplo de la variabilidad intraindividual es el hecho de que dentro del mismo individuo se puede encontrar fuertes contrastes en las hojas de copa y hojas de brote, hojas adultas y hojas jóvenes, hojas de luz y hojas de sombra (Jiménez *et al.,* 1996). Aparentemente, estos contrastes están asociados al ambiente de crecimiento de los distintos tejidos. La amplia distribución de *Q. ilex* incluye una inmensa variedad climas y sustratos que resultan en una gran diversidad de hábitats caracterizados por diversos grados de estrés. La presión selectiva generada por esta diversidad de hábitats puede provocar la existencia de un mosaico de poblaciones que difieren unas de otras en sus caracteres funcionales (García *et al.,* 1998; Gratani *et al.*, 2003; Sánchez-Vilas & Retuerto, 2007; Laureano *et al.*, 2008 y 2016).

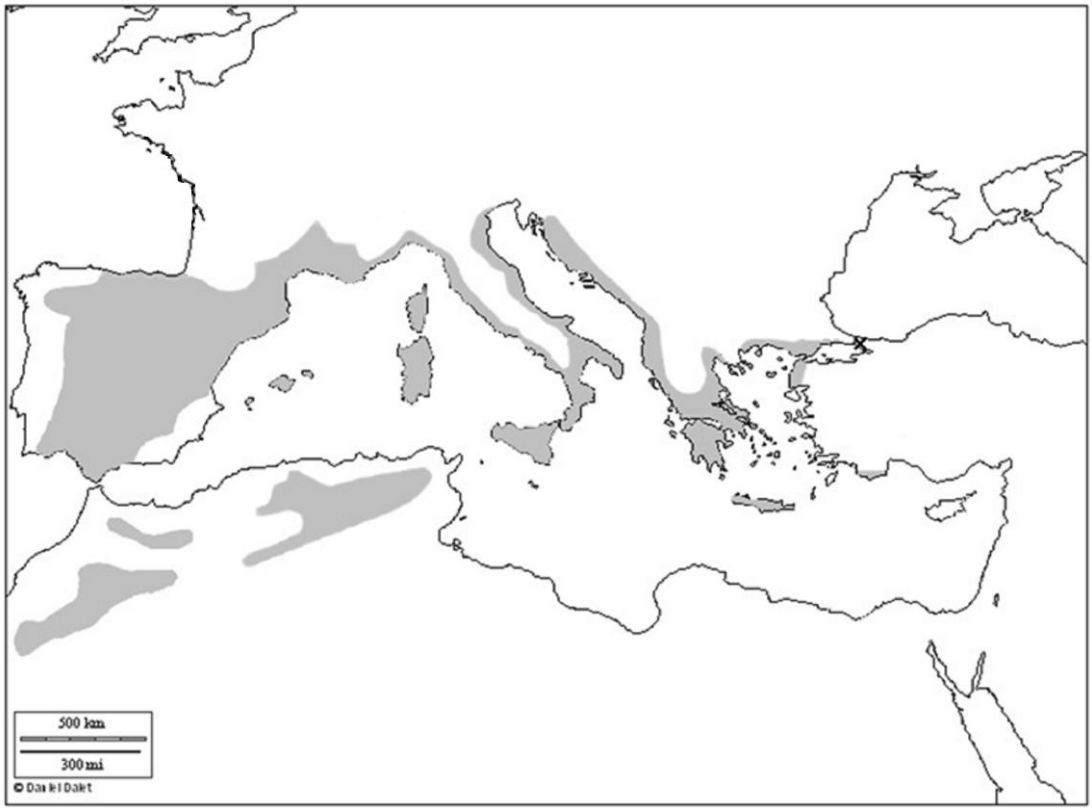

Figura 1. Área de distribución de *Q. ilex* en la Cuenca Mediterránea (modificado de Delzon *et al.*, 2013).

Este polimorfismo ha llevado a diversos autores a la conclusión de que existen dos subespecies de encina (véase más abajo), aunque hay otros que defienden que se trata de la misma especie pero con gran polimorfismo intraespecífico. Actualmente, Flora Ibérica reconoce dos subespecies distintas (Amaral Franco, 1990): *Quercus ilex* subsp. *ilex* L. y *Quercus ilex* subsp. *ballota* (Desf.) Samp. Asimismo, los estudios filogenéticos realizados en *Quercus ilex* (Lumaret *et al*., 2002), publicaron un mapa detallado de la distribución de los grupos filogenéticos que abarcan toda el área de distribución de esta especie. En dicho trabajo se comparan los tres morfotipos considerados, con los clorotipos observados en el árbol filogenético resultante. En general, hay buena consistencia entre los morfotipos y los clorotipos. En la región occidental del área de distribución se localiza el clorotipo denominado *rotundifolia*, que hace alusión a la subespecie denominada con el mismo nombre (o *ballota*) y que abarca la Península Ibérica y el Norte de África. El

clorotipo *ilex* se corresponde con la subespecie *ilex* localizada en la región oriental del área de distribución (Sur de Francia, Italia, Grecia). Aunque en estudios anteriores basados en caracteres morfológicos (Lebreton *et al*., 2001) se ha sugerido que las poblaciones del Levante español y del Ródano francés pertenecen a un morfotipo intermedio entre ambas subespecies (originado por hibridación y/o introgresión genética), según Lumaret *et al.* (2002), las poblaciones intermedias están más emparentadas con la subespecie *ballota* que con *ilex*; sugiriendo estos autores que los cambios morfológicos de la subespecie *ballota* observados en estas poblaciones intermedias son consecuencia de la adaptación a condiciones climáticas locales; lo que hace presuponer que las 12 poblaciones consideradas en el presente estudio pertenecen al clorotipo *ballota.*

El área de distribución de la subespecie *Quercus ilex ballota* se extiende desde los 43° 30′ N, hasta la costa oeste africana en Agadir (Marruecos) (30° 30′ N). Su rango de distribución altitudinal depende de la latitud. Así, en la zona africana esta especie aparece comúnmente desde el nivel del mar hasta los 2200 m (Rif y Atlas argelino); aunque puede alcanzar cotas más elevadas en el Atlas marroquí (2900 m). En la Península Ibérica aparece desde el nivel del mar hasta los 1500 m, aunque puede alcanzar los 2000 m. en ciertas localizaciones de Sierra Nevada. Su límite altitudinal disminuye hacia el Norte, de forma que en el norte de la Península Ibérica raramente se encuentra por encima de los 1200 m (Jiménez *et al*., 1996).

4. OBJETIVOS

El objetivo principal de esta Tesis es analizar la proyección biogeográfica de la variabilidad intraespecífica de la encina (*Q. ilex ballota*), utilizando para ello poblaciones procedentes de un gradiente latitudinal que abarca el rango de distribución completo de la

especie. En el estudio se han considerado tanto individuos creciendo en condiciones homogéneas (invernadero y cámaras de cultivo), como individuos creciendo en condiciones naturales.

De acuerdo con los argumentos expuestos más arriba, la hipótesis a testar es que, a pesar de lo contrastado de sus hábitats, las poblaciones nativas de la periferia del área de distribución deben presentar características fenotípicas (morfológicas y fisiológicas) similares entre sí y distintas de las que presentan las poblaciones procedentes del núcleo de su área de distribución. En la misma línea, las respuestas fenotípicas al estrés por parte de las poblaciones periféricas deberían ser convergentes, y –asimismo– difiriendo de las respuestas de las poblaciones nativas del núcleo del área de distribución.

Concretamente, se han abordado los siguientes objetivos específicos:

1. Evaluar la variabilidad interpoblacional de los caracteres morfológicos, a partir de datos obtenidos en individuos adultos creciendo en condiciones naturales, a lo largo de un gradiente latitudinal (Capítulo 1).

2. Analizar el patrón de diferenciación de los caracteres asociados al fruto, al crecimiento de las plántulas y a la distribución de sus fracciones, así como de las características del aparato fotosintético, en plántulas creciendo en condiciones homogéneas con objeto de detectar posibles patrones biogeográficos (Capítulos 1 y 2).

3. Analizar la variabilidad interanual de los patrones biogeográficos observados a lo largo de 4 años de estudio (Capítulo 1).

4. Analizar las relaciones entre las características morfológicas y fisiológicas consideradas en plántulas crecidas en cultivo controlado; tanto en condiciones libres de estrés como en condiciones de estrés ambiental (Capítulos 2 y 3).

5. Analizar la respuesta al de estrés (fumigación con ozono) a nivel fisiológico y morfológico en plántulas de encina nativas de 12 localidades que abarcan el rango latitudinal del área de distribución de la especie y su relación con su posición biogeográfica (Capítulo 3).

6. Analizar la evolución de las características del Fotosistema II (PSII) en respuesta a los cambios estacionales de temperatura e intensidad luminosa con objeto de cuantificar el grado de diferenciación intraespecífica y su posible relación con el clima de las localidades originarias de las poblaciones consideradas (Capítulo 4).

7. Determinar la plasticidad de los procesos fotoquímicos del PSII de 12 poblaciones de encina (Capítulo 4).

Con el propósito de cumplir estos objetivos, la presente Tesis Doctoral incluye cuatro capítulos. Todos ellos han sido escritos en inglés para su publicación en revistas científicas de ámbito internacional.

5. CONSIDERACIONES GENERALES

En cada uno de los capítulos de esta memoria se desarrollan los materiales, métodos y análisis estadísticos seguidos en sus apartados correspondientes; por lo que el objetivo de este apartado es esbozar de manera resumida las líneas comunes de los cuatro trabajos realizados.

5.1. Zonas de estudio

Para la realización del estudio, con objeto de recoger la máxima variación en lo que se refiere a los factores climáticos (humedad y temperatura), se seleccionaron 12 estaciones siguiendo la dirección Norte-Sur, de forma que quedasen representados los límites Norte y Sur del área de distribución y las poblaciones del interior del área.

Para ello se seleccionaron, por un lado, una serie de estaciones localizadas en la mitad Oeste de la Península Ibérica y Marruecos, que incluye las siguientes localidades (Figura 2): Astorga (León) (42° 27'), Aldeanueva de Figueroa (Salamanca) (41° 11') y El Pintado (Sevilla) (37° 59'); y las localidades marroquíes de Cherafate (Chaouen) (35° 05'), Faryata (Beni Mellal) (32° 23') y Lkoumss (Agadir) (30° 45'). En paralelo con esta serie, se seleccionó otro conjunto de estaciones localizadas al Este de la Península Ibérica y Sur de Francia, coincidiendo aproximadamente con la banda latitudinal definida por el eje Valle del Ródano (Francia) y la provincia de Almería: La Cadière-et-Cambo (Cambo) (43º 58') localizada en el límite Norte del área de distribución, Rojals (Montblanc) (41º 20'), Atzeneta del Maestrazgo (Atzeneta) (40º 15'), Alcoy (Font Roja) (38º 39'), Tabernas (Alhamilla) (36º 59') y Felix (36º 54'). La Tabla 1 resume la información sobre la vegetación acompañante al encinar y las características del sustrato de cada una de las estaciones. En la Figura 2 se presenta una panorámica de las distintas estaciones de muestreo y su localización en el área de distribución de la especie.

Las dos poblaciones ubicadas en los extremos del área de distribución son las catalogadas como poblaciones periféricas, cuyo punto extremo más septentrional se encuentra en el Languedoc francés (Cambo), y el extremo más meridional en la zona oeste del Alto Atlas de Marruecos (Agadir) (Figura 2). Las poblaciones de León y Felix, a priori,

también son consideradas como periféricas debido a su posición en el gradiente latitudinal. El resto de poblaciones se consideran como poblaciones núcleo en nuestro estudio.

5.2. Experimentos

El presente estudio se llevó a cabo a lo largo de los años 2009 a 2013. Cada año, la recolección de bellotas se realizó entre los meses de octubre y diciembre. En cada estación de muestreo se tomaron una media de 1000 bellotas procedentes de 20 árboles elegidos aleatoriamente y separados entre ellos al menos unos 30 metros para disminuir la probabilidad de muestrear individuos muy emparentados.

Los frutos cosechados se trasladaron al laboratorio donde se desechaban los podridos y los dañados por ataques de insectos. Los restantes se sometieron a un tratamiento fungicida para evitar el crecimiento de hongos antes de su siembra y se almacenaron en la nevera durante varios días. Antes de su siembra las bellotas fueron pesadas y marcadas individualmente para su posterior seguimiento tras la germinación. Finalmente, se seleccionaron unas 500 bellotas por población, incluyendo la mayor diversidad de tamaños posibles, y se sembraron en el invernadero del área de Ecología de la Universidad Pablo de Olavide (Sevilla) en bandejas con un sustrato de perlita.

Cada año, y aproximadamente un mes después de la germinación, se eligieron aleatoriamente alrededor de 100 plántulas por población y se sembraron en macetas con un sustrato formado por una mezcla de arena y perlita (3:1 v/v). Con estas plántulas se realizaron los experimentos que se describen en la presente memoria. Globalmente se realizaron las siguientes determinaciones:

- En cada estación de muestreo, se instalaron *dataloggers* para el registro de la temperatura ambiental y de la humedad relativa durante al menos 2 años. Se han realizado determinaciones estructurales y morfológicas (altura del árbol y de la copa, área basal del tronco a la altura del pecho, diámetro de las copas de los árboles y porcentaje de cobertura arbórea) en individuos adultos en algunas de las estaciones de muestreo. En las mismas localidades se midieron algunos caracteres morfológicos foliares (área y peso foliar individual, área específica foliar), así como otras determinaciones complementarias.

- Las plántulas de las distintas poblaciones creciendo en cámaras de cultivo se cultivaron en condiciones óptimas, en las que un grupo de cada población fueron sometidas a fumigación con ozono. En estas plántulas se midieron sus características morfológicas, caracteres foliares (área y peso foliar individual, área específica foliar) y estructurales (relación entre la fracción de biomasa aérea con respecto a las raíces, área foliar del individuo, área foliar del individuo en relación a su peso total y peso foliar del individuo en relación a su peso total); así como su tasa específica de crecimiento. Además, se cuantificaron variables fisiológicas asociadas al intercambio de gases y a la emisión de fluorescencia de la clorofila *a*. Algunas de estas determinaciones se realizaron también en condiciones de invernadero (ausencia de control de la temperatura, humedad atmosférica y condiciones luminosas).

Tabla 1. Vegetación asociada y sustrato de las 12 estaciones de muestreo del estudio.

	Agadir	**Beni Mellal**	**Chaouen**	**Felix**	**Alhamilla**	**Sevilla**	**Font Roja**	**Atzeneta**	**Salamanca**	**Montblanc**	**León**	**Cambo**
Vegetación asociada	*Chamaerops humilis, Ononis sp.*	*Chamaerops humilis, Cistus salviifolius, Pistacia lentiscus*	*Arbutus unedo, Olea europea sylvestris, Pistacia lentiscus, Smilax aspera*	*Chamamerops humilis, Pistacia lentiscus*	*Cistus albidus, Cistus ladanifer, Daphne gnidium*	*Chamaerops humilis, Cistus ladanifer, Genista hirsuta, Quercus suber*	*Acer opalus sp. granatense, Crataegus monogyna, Fraxinus ornus, Quercus faginea, Rosa sp.*	*Erica arbórea, Pinus halepensis, Rhamnus alaternus, Rosmarinus officinalis,*	*Cistus ladanifer, Crataegus monogyna, Rosa sempervirens, Pinus pinaster*	*Adenocarpus sp. Juniperus oxycedrus, Pinus sylvestris*	*Cistus ladanifer, Daphne gnidium, Lavandula sp.*	*Cedrus atlantica, Corilus avellana, Hedera sp., Pinus sylvestris, Quercus pubescens*
Geomorfología	Pendiente	Llana	Pendiente	Pendiente	Pendiente	Ondulada	Pendiente	Pendiente	Llano	Pendiente	Llano	Pendiente
Roca madre	Caliza	Caliza	Caliza	Caliza	Contacto Micacitas-Dolomías	Pizarra	Caliza	Caliza	Conglomerado ácido (raña)	Caliza	Conglomerado calizo (raña)	Caliza
Clasificación FAO (1991)	Litosol	Litosol, Rendsina	Litosol	Litosol	Ranker	Cambisol	Rendsina	Cambisol	Litosol	Cambisol	Litosol	Cambisol

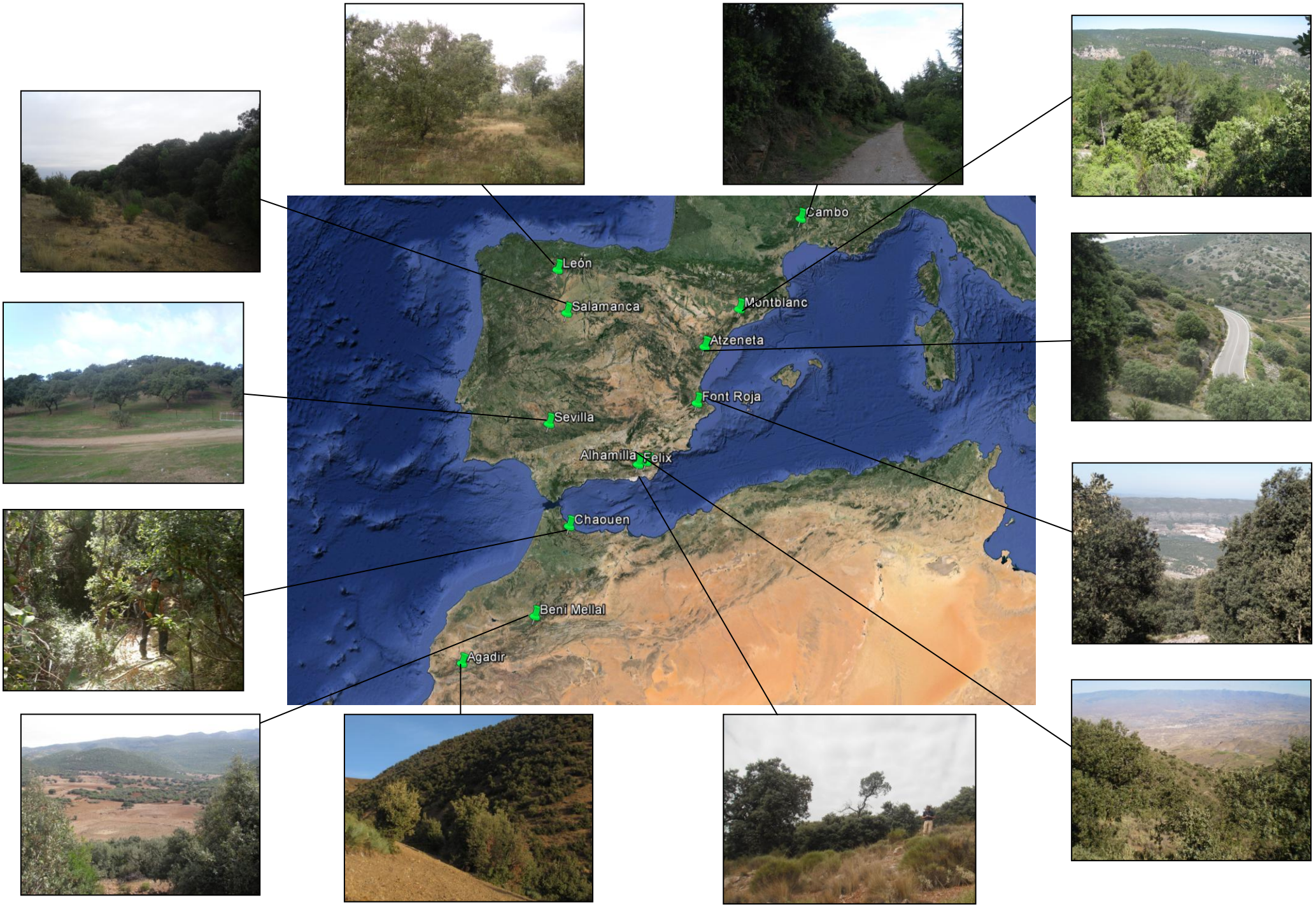

Figura 2. Localización de las 12 estaciones de muestreo a lo largo del área de distribución de *Quercus ilex ballota*.

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II. CAPÍTULO 1

Range-wide variation in life history phenotypes: spatio-temporal plasticity across the latitudinal gradient of the evergreen oak *Quercus ilex*

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Range-wide variation in life history phenotypes: spatio-temporal plasticity across the latitudinal gradient of the evergreen oak *Quercus ilex*

Ana García-Nogales, Juan C. Linares, Raquel G. Laureano, José I. Seco, José Merino

1. ABSTRACT

Stressful environments, like those at the limits of species ranges, are closely associated with the selective establishment of particular traits, yet the mechanisms behind this relationship are poorly understood. Our main aim was to investigate spatial and temporal phenotypic plasticity related to environmental conditions and biogeographical origin. The study area is a latitudinal gradient along Iberian Peninsula and Morocco, western Mediterranean Basin. Here we investigate the intra-specific variability of the Mediterranean oak *Quercus ilex* subsp. *ballota*, covering most of its latitudinal range. Acorns were collected in the years 2009, 2011, 2012 and 2013 from natural populations and were grown under controlled conditions. We measured acorn mass and germination dynamics, as well as seedling morphology. Field data showed significant differences in stand structure among populations. The southernmost population presented the smallest trees, followed by the northernmost population. Core-range trees registered higher values for leaf-nitrogen concentration. Leaf size showed latitudinal variation, with the lowest values at the northern range limit. In addition, the germination pattern also significantly differed among populations, showing a latitudinal pattern of variation (i.e., the southern populations germinated earlier). Fresh acorn mass showed significant within- and among-population differences, displaying a range margin pattern (i.e. similarities between the edge populations). Furthermore, seedling total weight and

biomass allocation also followed the same pattern. The models explaining the most part of the trait variance were related mainly to climatic variables (reflecting temporal variability), while the random effect of the models (population) showed a range margin pattern with lower values in edge populations compared with core populations. Similar phenotypic traits among individuals native to the periphery of the distribution range were found despite the temporal variability, suggesting adaptation to stressful environments at the range edges.

Key-words: acorns, biogeography, climatic gradient, germination, local adaptation, Mediterranean evergreen, morphological traits, phenotypic plasticity, structural traits.

2. INTRODUCTION

Plants live in ever-changing environments and must adapt using different strategies to modify their growth and organs in response to different environmental signals. Stressful environments, such as those located at the distribution-range limits of a species, influence the expression of phenotypes in plant species with high phenotypic plasticity (Vergeer & Kunin, 2013; Jump & Peñuelas, 2005), yet the mechanisms behind this relationship are poorly understood (Sexton *et al.*, 2009). Several recent studies have significantly furthered our understanding of the important role that stress-induced variation plays in plant adaptive capacity to cope with changing environments (Vergeer & Kunin, 2013; Ramírez-Valiente *et al.*, 2014; Bussotti *et al.*, 2015; Matías & Jump, 2015).

It is widely acknowledged that different populations within a given plant species may inhabit contrasting environments. Stress-induced variation, related to such contrasting environments, should provide a selective pressure on plant developmental systems (allocation patterns in plant growth, as well as plant morphological traits). Such stress-induced variation might remain as a constitutive plant trait under non-stressful growth conditions, supporting non-plastic phenotypic assimilation of the response to a stressor. As a result, ecotypes or ecological races may appear (Laureano *et al.*, 2008; Bruschi, 2010; Arend *et al*., 2011; Hatziskakis *et al*., 2011).

Phenotypic plasticity and local adaptation can both influence the ability of a plant to adapt to its environment (Bussotti *et al*., 2015). In changing environments, stress-induced phenotypic changes may be often directional, accounting for similarity among individuals native to stressful habitats, such as those bordering range limits (Li *et al*., 1998; Matías & Jump, 2015). It has been suggested that the increasing phenotypic plasticity might be a common property of locally-adapted plant populations native to stressful environments (Ghalambor *et al.*, 2007).

Stress-induced selection of higher phenotypic plasticity may result in spatial patterns of particular traits across the distribution area of a given species (Bruschi, 2010; Ramírez-Valiente *et al*., 2010; Bonito *et al*., 2011). These geographical responses, together with both the complexity of developmental systems and stressor recurrence, might facilitate phenotypic and/or genetic assimilation of stress-induced effects. For instance, cork oak (*Quercus suber* L.) populations have shown a pattern of declining drought resistance as latitude increases (Ramírez-Valiente *et al*., 2009), while low-temperature tolerance increases with latitude (Aranda *et al*., 2005). In short, the

expression of individual phenotypic plasticity to stress provides a link between individual adaptive capacity and stressful environments (Davis & Shaw, 2001).

Studies accounting for phenotypic plasticity over the entire distribution range of species, including the stressful environments typical of the range edges, may provide useful ecological and biogeographical information under a global change scenario; however, such studies are still scarce. Uribe-Salas *et al*. (2008) stated that the latitudinal variation observed in the Mexican oak *Quercus rugosa* Née foliar traits might result not only from the environmental variation related to latitude, but also from geographical processes such as genetic drift or local isolation. Furthermore, several studies have pointed out the contrasting characteristics among populations belonging to the edges and the core of the range (Hampe & Petit, 2005; Sexton *et al.*, 2009; Matías & Jump, 2015). Such heterogeneity has been related to contrasting and likely nonlinear, changing environmental conditions, where stressful characteristics at range margins boost phenotypic plasticity (Vergeer & Kunin, 2013).

Plant species able to grow over a wide range typically show significant variability in several physiological and morphological functional traits (García *et al*., 1998; Ramírez-Valiente *et al*., 2014), providing useful experimental models to investigate the adaptive value of such variability. The genus *Quercus* is found in temperate, Mediterranean and tropical climates, usually in woodland or forest, but sometimes in shrublands (Kleinschmit, 1993; Nixon, 2006), showing high intra- and inter-population variability (Uribe-Salas *et al*., 2008). Morphological and functional traits such as seed size and germination rates, seedling growth, and biomass-allocation patterns provide reliable information about the capacity of plants to adapt to contrasting environmental conditions. For instance, *Quercus* seed size might be significantly related

to population recruitment under stressful conditions (Quero *et al*., 2007; Ramírez-Valiente *et al*., 2009; Andivia *et al*., 2012).

Holm oak (*Quercus ilex* L.) is a common evergreen tree species distributed throughout the Mediterranean Basin, growing in a wide range of soils and climate conditions (Barbero *et al*., 1992; García *et al*., 1998; Gratani *et al*., 2003; Sánchez-Vilas & Retuerto 2007; Laureano *et al*., 2008 and 2013). Genetic analyses support an east-west differentiation (Lumaret *et al*., 2002; López de Heredia *et al*., 2007), as well as high haplotype variability (Lumaret *et al*., 2002; Jiménez *et al*., 2004).

Here, we investigate the sub-species *Quercus ilex* subsp. *ballota* (Desf.) Samp, over its whole distribution range, from Montpellier (south of France) to the west coast of Africa at Agadir (Morocco). Our study attempts to disentangle the contribution of environmental conditions (latitude-related climate gradient) and range structure on spatio-temporal population variability. This general framework might illustrate that species variability in response to environmental changes likely relies on the stress-induced variations showed by populations native to range margins. Specifically, we: i) investigate whether there is a biogeographical pattern of differentiation in acorn characteristics (weight, germination rate) and resulting seedling growth rate and biomass allocation patterns under common growth conditions; ii) analyse the inter-annual variability of these biogeographical patterns, accounting for short-term changing environmental conditions.

3. MATERIAL AND METHODS

3.1. Sampling areas

We studied five populations of *Quercus ilex* subsp. *ballota* (Desf.) Samp. (hereafter referred to as *Q. ilex*) located along a latitudinal gradient in the west of its distribution range (Table 1 and Fig. 1). Sampling was focused on remnant, or near-to-natural, *Q. ilex* populations, where there is a low-intensity of human perturbation with no apparent differences among populations. The latitudinal gradient covers the main range of continental climatic conditions of the *Q. ilex* distribution range. Two locations were considered to be peripheral populations at the boundary of the species' distribution range (northern Spain [NS] and southern Morocco [SM]) and three belong to the core distribution range (central Spain [CS], southern Spain [SS], and central Morocco [CM]). Mean annual temperature, radiation, potential evapotranspiration and aridity index generally increased from north to south of the distribution range (Table 1 and Fig. 1a). The peripheral populations seem to withstand higher levels of climatic stress than the core ones: The northernmost location was characterized by the lowest values of minimum temperatures in winter with several days registering temperatures below 0ºC, and also displayed the highest differences between maximum and minimum temperatures along the year; in contrast the southernmost population registered the highest values for maximum temperatures, solar radiation, potential evapotranspiration, and aridity. For further details about climatic data and the characteristics of study locations (soil type, associated vegetation, etc.) see Appendix S1 in Supporting Information.

Table 1. Location and climatic data for the five *Quercus ilex* study sites in Spain and Morocco. Pa, annual rainfall; Ps, summer rainfall; T, mean annual temperature; Tmax, mean maximum temperature in summer; Tmin, mean minimum temperature in winter; 0ºC days, mean number of days over 2012 and 2013 in which the minimum temperature was less than 0ºC; Rad, mean solar radiation; PET, corrected potential evapotranspiration; Water balance, Pa-PET; De Martonne AI, De Martonne's aridity index, -(Pa/(T+10)). The locations in the table are given in order of increasing latitude from left to right.

Location Code	**SM**	**CM**	**SS**	**CS**	**NS**
Location	Agadir	Beni Mellal	Sevilla	Salamanca	Leon
Latitude (N)	30°45'	32°23'	37°59'	41°11'	42°27'
Longitude (W)	8°44'	6°12'	5°57'	5°30'	5°58'
Elevation (m.a.s.l.)	1553	850	347	886	871
Pa (mm)	309	443	574	403	505
Ps (mm)	19.9	18.8	19.8	49.0	59.1
T (ºC)	21.3	17.2	17.1	10.8	10.8
Tmax (ºC)	33.6	27.1	33.5	25.2	29.7
Tmin (ºC)	10.6	8.8	5.7	0.7	-0.5
0ºC days (2012-2013)	0	0	0	18	30
Rad (W m^{-2})	695	681	622	580	566
PET (mm)	1135	849	890	653	654
Water balance (mm)	-826	-405	-316	-250	-149
De Martonne AI (unitless)	-9.9	-16.3	-21.2	-19.4	-24.3

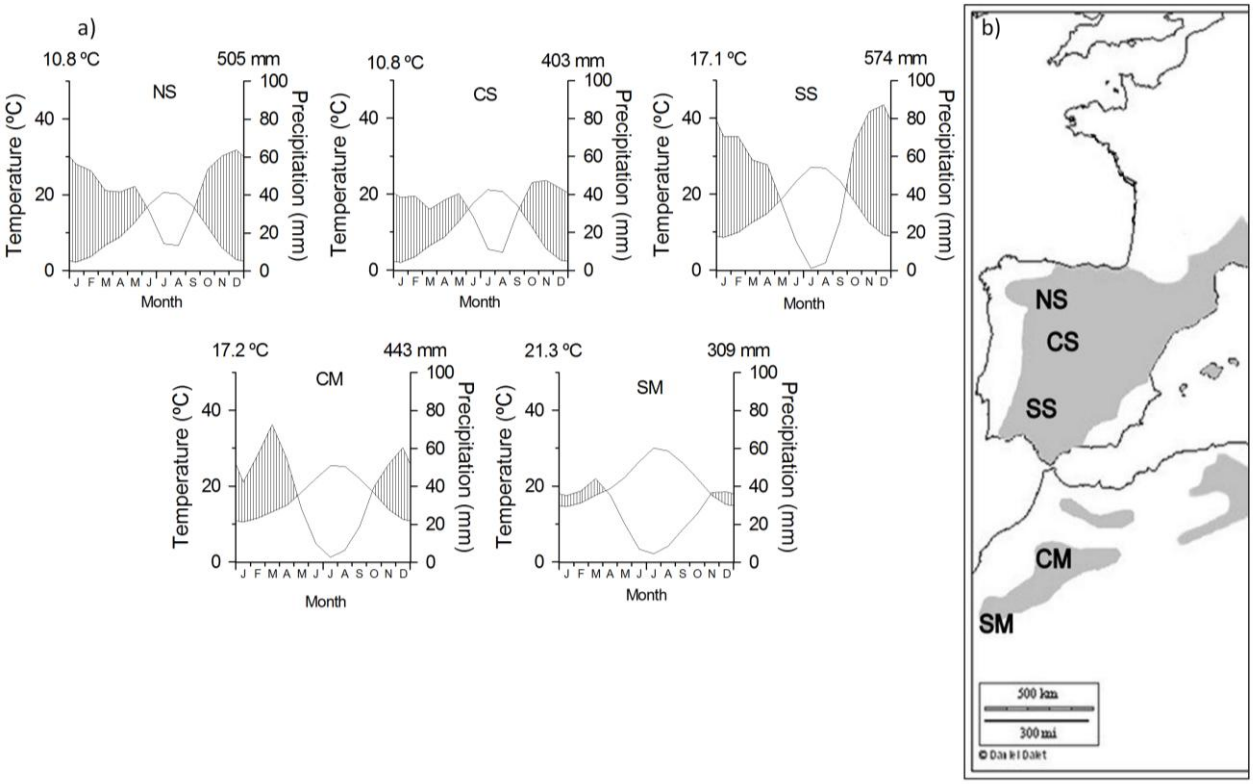

Figure 1. a) Average monthly temperature (black line) and rainfall (vertical lines) based on the interpolated E-OBS v10.0 and CRU TS 3.22 climate database and on local climate data for each population of *Quercus ilex* studied in Spain and Morocco (see Appendix S1 in Supporting Information). Location code: NS: northern Spain; CS: central Spain; SS: southern Spain; CM: central Morocco; SM: southern Morocco. Data are means for the 1950-2014 period. b) Locations of the sampled populations in the western part of *Q. ilex* distribution range (map modified from Delzon *et al*., 2013).

3.2. Field Sampling

Acorn harvesting took place during the autumn of 2009, 2011, 2012 and 2013. Every year, a mean of 1000 acorns per population were collected from 20 randomly assigned trees separated by at least 30 m to reduce the chance of sampling related trees. For the same trees, we carried out the stand-structural characterization of the forest for each location. Tree and crown height were estimated trigonometrically using a tape measure, and the tree basal area was estimated by measuring the tree diameter at the breast height (dbh). The crown diameter for each tree and the canopy cover percentage for each study site were quantified from aerial images (GOOGLE EARTH 7.1) and using image-analysis

software (IMAGE J 1.45). Mature leaves from the previous growth period were collected from the 20 assigned trees per population to quantify leaf traits: fresh leaf-blade size (LS, [cm^2], measured using the image-analysis software), leaf dry weight (LW, [g], determined by weighing individual leaves) and specific leaf area (SLA, [$cm^2\ g^{-1}$], calculated as LS/LW). In addition, a subset of mature leaf samples were oven dried for 48 h at 60ºC and were ground to a fine powder. Leaf N and C concentrations were measured in these leaves using an elemental analyser (LECO Corporation, St. Joseph, Michigan, USA) and the results were expressed as mg g^{-1}.

3.3. Culture and growth conditions

For each study year, acorns were pooled by population, labelled and weighed to determine their fresh mass (acorn mass ± 0.001 g). Labelled acorns were placed in trays with perlite substrate for germination under greenhouse conditions at Pablo de Olavide University, Seville, Spain (37°21'3N, 5°56'7W, 15 m.a.s.l.). When germination started, the number of germinated seeds per population was recorded for at least 40 days to determine the germination rate. Every year, one month after germination, around 100 seedlings per population were selected and sown in 1-litre PVC pots filled with a 3:1 sand:perlite mixture. The seedlings were grown for two months in a controlled-environment greenhouse (at a day/night mean temperature of 19/11ºC, a mean RH of 62%, and a mean photon flux density of 300 µmol $m^{-2}s^{-1}$).

When seedlings were roughly 10 cm tall, a mean of 35 plants per population were randomly selected each year. Fifteen seedlings per population were used to estimate the individual mean dry weight of each population (initial weight). Pooled seedlings from the five populations (a mean of 20 individuals per population) were placed in three 1 m^3 growth chambers under the following conditions: day/night

temperature of 25/20ºC, day/night RH of 56/80%, photosynthetic photon flux density (PPDF) of 400 µmol $m^{-2}s^{-1}$ at plant height, and 11-h photoperiod. Plants were watered twice daily with tap water using a drip system to ensure adequate water availability. *Hoagland* nutritive solution (modified from Hoagland & Arnon, 1950) was added twice weekly to offset the lack of nutrients in the substrate. To minimize the chamber effect, plants were rotated both inside chamber and between chambers (once weekly).

After three months of growth, seedlings were separated into stems, leaf and root fractions, oven dried at 70ºC and weighed (final weight). Just before seedlings were harvested, three fresh leaves were collected to measure mean leaf-surface area using image analysis software. The relative growth rate (RGR, mg $g^{-1}day^{-1}$) of each individual of a particular population was calculated by subtracting the initial weight of that population from the final weight of that particular individual, based on the three-month period. The following seedling structure traits also were accounted: specific leaf area (SLA), fresh leaf-blade size (LS), leaf dry weight (LW), shoot to root ratio (S/R), total leaf surface (LA), total leaf-area to plant weight (LAR) and total weight of leaves to plant weight (LWR). SLA was calculated as the ratio LS/LW, whereas S/R was determined as shoot to root weight for each seedling. LA per seedling was calculated by weighing the total number of leaves and multiplying by average individual leaf area. LAR for each seedling was calculated by LA by total seedling weight; while LWR was estimated by dividing the total leaf weight by total seedling weight.

3.4. Statistical analyses

Trait data collected in the field were analysed by a one-way ANOVA using population as the independent variable. The variability of acorn and seedling traits was assessed by

a factorial ANOVA including *Year* and *Population* as independent variables, as well as its interaction. Data were transformed when necessary to meet the assumptions of ANOVA. When differences were relevant, an *a posteriori* test was conducted (Tukey's *post hoc* HSD) to make individual comparisons at the population level. The results were analysed using statistical software STATISTICA 7.0.

Following Linares *et al.* (2012), sigmoidal curves were fitted to data recorded during the germination:

$$y = \frac{a}{1 + e^{-(\frac{x - x_0}{b})}}$$

where *y* is the accumulated germination percentage (%), *x* is the time (days). The fitted model provides three estimated parameters: *a* is the asymptote that represents the final germination percentage, *b* is the maximum slope of the sigmoid function and *xo* represents the day when the slope is maximum –this value is equivalent to the time to 50% germination (T_{50})–. For further statistical analyses to test differences for germination dynamics see Appendix S2 in Supporting Information (S2.1., Table S2.1.).

To model the variability of inter-annual acorn traits, we applied linear mixed-effects models (GLMM) using the R package 'nlme' 3.1-127 (Pinheiro *et al.*, 2016). These models included climatic variables from 2009, 2011, 2012, and 2013 as fixed factors and population as a random factor. The climatic variables were selected using an information-theoretic approach to multi-model selection, based on Akaike information criterion corrected for small sample sizes (AICc). The model selected for each acorn trait was the one which included the lowest amount of explanatory variables among those with the lowest AICc (Burnham & Anderson, 2002). To model seedling total weight, RGR and S/R, we applied the same models but using acorn mass and seedling-

structure traits as fixed factors and population as a random factor. Here, the factor year was not included because we assume that inter-annual variability in acorn traits incorporate this information. Finally, when the random factor values indicated population structure, linear-regression analyses were used to test the occurrence of relationship between random factors for each population and latitude (geographic location) or climatic variables.

4. **RESULTS**

4.1. Field variables

Under field conditions, populations showed several differences in stand-structural and leaf traits (Table 2). The southernmost population (SM) presented the significantly smallest trees ($P < 0.001$), with the lowest values of tree height, crown diameter, and crown height; this was followed by the northernmost population (NS). On the contrary, the SS and CM populations had the biggest trees. The CM population presented the significantly highest values for leaf nitrogen concentration (16.1 ± 0.5 mg g^{-1}; $P < 0.001$) measured in mature leaves (followed by SS and CS); while the SM and NS populations had the lowest values (11.8 ± 0.5 and 12.9 ± 0.4 mg g^{-1}, respectively). Both leaf-blade size (LS) and leaf weight (LW) displayed a clear latitudinal variation ($P < 0.001$), with the lowest values in NS (2.81 ± 0.09 cm^2 and 0.060 ± 0.002 g, respectively). All the populations had similar values of SLA.

4.2. Acorn traits

The analysis of variance for fresh acorn mass, germination time (T_{50}) and percentage germinated after 40 days (accumulated germination) showed that the main effects of *Population* and *Year* and their interaction were large and significant ($P < 0.001$, Table 3

Table 2. Mean values (±SE) of the stand variables of *Quercus ilex* measured in field conditions for each location in Spain and Morocco. Significance from one-way ANOVA is noted in bold when $P < 0.05$. Different letters indicate significant differences among locations following Tukey HSD test. The populations in the table are given in order of increasing latitude from left to right. Abbreviations: LS = leaf-blade size; LW = leaf dry mass; SLA = specific leaf area. The last column denotes whether the pattern of variation for field traits among populations is range margin (RM), latitudinal (L) or there is not a clear pattern of variation (-).

Code	SM	CM	SS	CS	NS	*P*	Pattern of variation
Tree height (m)	3.00 ± 0.04 a	6.52 ± 0.13 b	7.16 ± 0.17 b	5.19 ± 0.10 ab	5.05 ± 0.15 ab	**<0.001**	RM
Tree basal area (m^2)	0.073 ± 0.004 ab	0.092 ± 0.004 ab	0.105 ± 0.003 b	0.068 ± 0.004 ab	0.042 ± 0.002 a	**<0.05**	RM
Crown diameter (m)	3.17 ± 0.02 a	5.26 ± 0.08 b	6.78 ± 0.11 c	5.89 ± 0.10 bc	4.87 ± 0.07 b	**<0.001**	RM
Crown height (m)	3.00 ± 0.04 a	6.05 ± 0.13 b	5.66 ± 0.12 b	4.97 ± 0.10 b	4.68 ± 0.14 ab	**<0.001**	RM
Canopy cover (%)	23.4 ± 5.1 a	51.5 ± 3.7 b	34.8 ± 3.3 a	65.7 ± 3.5 b	56.7 ± 2.8 b	**<0.001**	-
Leaf [C] (mg g^{-1})	486 ± 2.6 b	458 ± 2.2 a	466 ± 2.0 a	465 ± 2.1 a	466 ± 2.1 a	**<0.001**	-
Leaf [N] (mg g^{-1})	11. 8 ± 0.5 a	16.1 ± 0.5 d	14.5 ± 0.3 c	13.9 ± 0.3 bc	12.9 ± 0.4 ab	**<0.001**	RM
LS (cm^2)	5.54 ± 0.20 c	5.58 ± 0.30 c	3.91 ± 0.17 b	3.41 ± 0.12 ab	2.81 ± 0.09 a	**<0.001**	L
LW (g)	0.123 ± 0.004 c	0.118 ± 0.006 c	0.087 ± 0.003 b	0.071 ± 0.003 ab	0.060 ± 0.002 a	**<0.001**	L
SLA (cm^2 g^{-1})	45.3 ± 0.6	46.9 ± 0.7	44.7 ± 0.7	48.1 ± 0.7	46.9 ± 0.7	n.s.	-

and Table S.2.2 in Appendix S2). Overall, the values of these three variables showed a latitudinal pattern (Table 4), CM being the population with the largest acorns and the highest values for germination time and accumulated rate (5.42 ± 0.09 g, 15.7 ± 2.5 days and 90.2 ± 3.9 %, respectively), and the lowest ones in the northernmost population (NS) (2.06 ± 0.04 g, 32.8 ± 5.3 days, 53.5 ± 16.2 %). The southernmost population (SM) displayed low values for acorn mass, as well as intermediate values for

germination time and accumulated rate, with respect to the other populations (Table 4). Fig. 2 shows panels with germination curves over time for the five populations considered in the four years of study. There was marked variation in the germination curves for each population, indicating high variability among years within populations. The germination rate in the 2011 experiment was extremely low in the NS population (8 ± 0.2 %). The SM population was not used in the 2012 experiment since acorn production for this population in that year was extremely low.

Table 3. Factorial ANOVA summary (F values) of acorn and seedling traits of *Quercus ilex* form Spain and Morocco regarding *Population* and *Year* as fixed factors, as well as its interaction, for the five populations considered cultivated in growth chambers. Asterisks denote significant differences from ANOVA (*: P < 0.05, **: P < 0.01, ***: P < 0.001). Abbreviations: T_{50} = time to 50% germination; LS = leaf-blade size; LW = leaf dry mass; SLA specific leaf area; RGR = relative growth rate; LA = total leaf surface; LAR = leaf-area ratio; LWR = leaf-weight ratio; S/R = shoot to root ratio.

	F for fixed sources of variation		
Trait	*Population*	*Year*	*Pop x Year*
Num DF	4	2	8
Acorn mass	287.9 ***	46.7 ***	19.5 ***
T_{50}	99.1 ***	35.4 ***	23.0 ***
Accumulated germination	164.6 ***	21.2 ***	85.8 ***
LS	27.9 ***	6.0 **	2.7 **
LW	19.6 ***	2.3	3.8 ***
SLA	12.7 ***	5.3 **	2.4 *
Leaves	16.3 ***	4.2 *	4.7 ***
Shoot	20.4 ***	8.0 ***	4.6 ***
Roots	42.2 ***	64.9 ***	6.9 ***
Wtotal	35.3 ***	34.3 ***	6.1 ***
RGR	0.5	46.5 ***	0.3
LA	16.1 ***	4.4 *	3.4 **
LAR	13.3 ***	76.9 ***	1.0
LWR	8.2 ***	84.2 ***	1.0
S/R	5.1 ***	36.0 ***	0.9

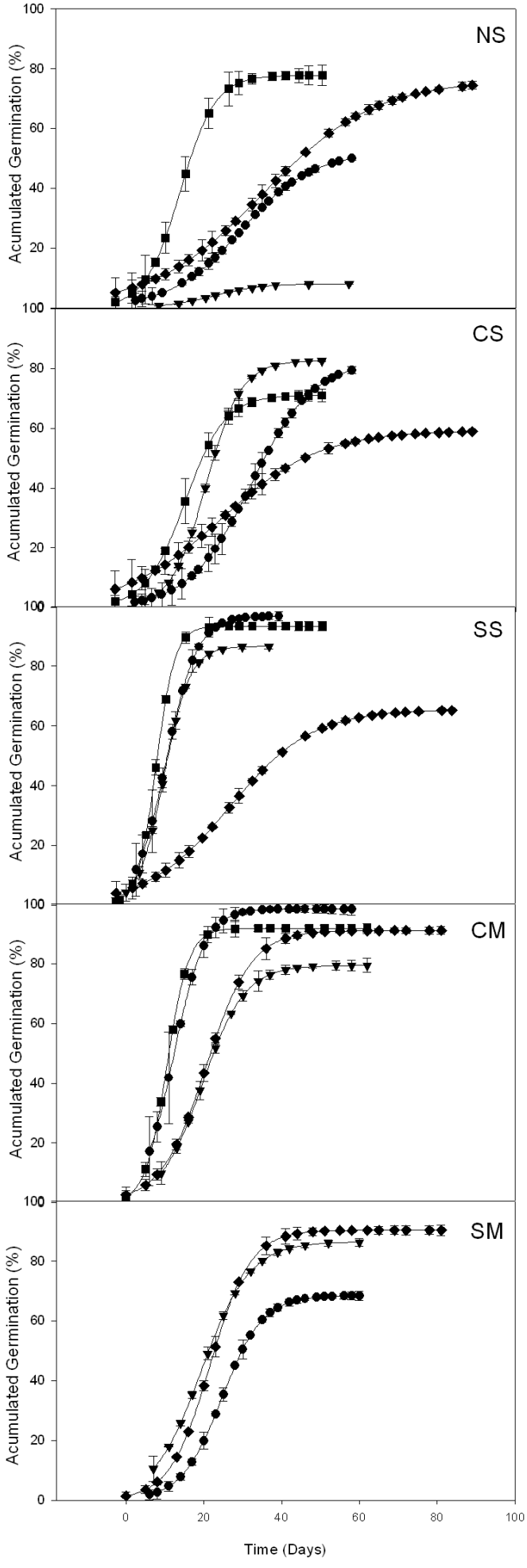

Figure 2. Germination rate measured in *Quercus ilex* acorns from five locations in Spain and Morocco (location code: NS: northern Spain; CS: central Spain; SS: southern Spain; CM: central Morocco; SM: southern Morocco) for four different years of study cultivated under greenhouse conditions: 2009 (circle), 2011 (triangle), 2012 (square), 2013 (diamond). Error bars represent the standard error; lines represent the fitted Gomperz function.

Table 4. a) Average and annual mean (±SE) values (2009, 2011, 2012, and 2013) of *Quercus ilex* acorn fresh mass for each population studied in Spain and Morocco. Different letters indicate significant differences among locations in Tukey's HSD test. b) Annual values of T_{50} (time to 50% germination) and percentage germinated after 40 days (accumulated germination) were calculated from parameters of the Gompertz functions fitted to the datasets of cumulative germination. Different letters indicate statistical differences among locations. The populations in the table are given in order of increasing latitude from left to right. The last column denotes if the pattern of variation for acorn traits among populations is range margin (RM) or latitudinal (L).

a) *Acorn fresh mass (g)*	SM	CM	SS	CS	NS	*P*	Pattern of variation
Average	**3.10 ± 0.07 b**	**5.42 ± 0.09 e**	**4.51 ± 0.08 d**	**3.76 ± 0.07 c**	**2.06 ± 0.04 a**	**<0.001**	**RM**
2009	3.13 ± 0.11 b	6.87 ± 0.19 e	5.36 ± 0.21 d	4.45 ± 0.14 c	2.21 ± 0.06 a	<0.001	
2011	3.20 ± 0.11 b	5.14 ± 0.21 d	5.39 ± 0.22 d	4.47 ± 0.17 c	1.29 ± 0.06 a	<0.001	
2012	-	5.25 ± 0.12 d	3.85 ± 0.11 c	3.44 ± 0.12 b	2.19 ± 0.06 a	<0.001	
2013	2.99 ± 0.13 a	4.61 ± 0.23 b	4.30 ± 0.14 b	2.95 ± 0.13 a	2.38 ± 0.08 a	<0.001	
T_{50} (days)	**SM**	**CM**	**SS**	**CS**	**NS**	***P***	**Pattern of variation**
b) **Average**	**21.8 ± 0.2**	**15.7 ± 2.5**	**18.9 ± 5.1**	**30.2 ± 4.1**	**32.8 ± 5.3**	**-**	**L**
2009	24.6 ± 0.2 c	12.2 ± 0.5 a	15.1 ± 0.5 b	40.8 ± 0.8 e	37.8 ± 0.7 d	<0.001	
2011	19.3 ± 0.3 b	19.6 ± 0.4 b	14.5 ± 0.3 a	27.3 ± 0.2 c	29.7 ± 0.6 d	<0.001	
2012	-	10.5 ± 0.2 a	12.1 ± 0.1 b	21.0 ± 0.8 d	19.4 ± 0.9 c	<0.001	
2013	21.6 ± 0.2 b	20.6 ± 0.3 a	34.1 ± 0.6 d	31.7 ± 1.1 c	44.2 ± 1.1 e	<0.001	
Acumulated germination (%)	**SM**	**CM**	**SS**	**CS**	**NS**	***P***	**Pattern of variation**
Average	**81.7 ± 6.7**	**90.2 ± 3.9**	**85.6 ± 7.0**	**73.9 ± 5.6**	**53.5 ± 16.2**	**-**	**L**
2009	68.4 ± 0.6 b	98.3 ± 1.4 d	96.7 ± 1.6 d	82.5 ± 2.3 c	52.0 ± 1.1 a	<0.001	
2011	86.5 ± 0.9 d	79.4 ± 0.9 b	86.6 ± 1.1 d	82.6 ± 0.6 d	8.0 ± 0.2 a	<0.001	
2012	-	91.8 ± 0.6 c	93.4 ± 0.6 d	71.1 ± 1.5 a	77.8 ± 1.9 b	<0.001	
2014	90.3 ± 0.5 d	91.1 ± 0.5 de	65.6 ± 0.7 b	59.2 ± 1.0 a	76.1 ± 1.4 c	<0.001	

4.3. Plant structure and growth rate

In general, plant structure and the leaf traits considered displayed highly significant differences among populations and among years within populations; whereas its interaction tended to be significant but small (Table 3 and Table S.2.2 in Appendix S2).

The separate weights of the seedling organs (leaves, shoot and roots), as well as the total weight, showed the same latitudinal pattern described above (Table 5), with maximum values in the CM population and minimum values in the NS one. Furthermore, as before, the SM population exhibited lower values of seedling biomass

than expected given its latitude and they were similar to those from the northernmost population (NS). Total leaf surface (LA) followed a similar pattern to the seedling organs, although differences among populations were less significant. The remaining leaf traits considered (LS and LW) showed a clear latitudinal decrease.

Table 5. Mean values (±SE) of morphological variables measured in *Quercus ilex* seedlings (in four different study years) from the five populations in Spain and Morocco considered cultivated in growth chambers. Significance from one-way ANOVA is noted in bold when $P < 0.05$. Different letters indicate significant differences among locations in Tukey's HSD test. The populations in the table are given in order of increasing latitude from left to right. Abbreviations: LS = leaf-blade size; LW = leaf dry mass; SLA = specific leaf area; RGR = relative growth rate; LA = total leaf surface; LAR = total leaf-area to plant weight; LWR = total weight of leaves to plant weight; S/R = shoot to root ratio; n = number of seedlings considered. The last column denotes if the pattern of variation for seedling traits among populations is range margin (RM) or latitudinal (L).

	SM	**CM**	**SS**	**CS**	**NS**	**P**	**Pattern of variation**
LS (cm^2)	8.12 ± 0.29 c	7.75 ± 0.26 c	6.00 ± 0.18 b	6.01 ± 0.19 b	4.84 ± 0.15 a	**<0.001**	L
LW (g)	0.114 ± 0.004 c	0.113 ± 0.004 c	0.097 ± 0.003 b	0.094 ± 0.003 b	0.073 ± 0.002 a	**<0.001**	L
SLA (cm^2g^{-1})	71.8 ± 1.2 d	68.8 ± 0.9 c	62.4 ± 0.7 a	64.5 ± 0.6 ab	66.9 ± 0.7 bc	**<0.001**	RM
Leaves (g)	2.06 ± 0.12 b	2.63 ± 0.10 c	2.32 ± 0.11 bc	2.29 ± 0.10 bc	1.47 ± 0.08 a	**<0.001**	RM
Shoot (g)	2.88 ± 0.17 b	3.96 ± 0.15 c	3.25 ± 0.16 b	3.43 ± 0.16 bc	2.03 ± 0.10 a	**<0.001**	RM
Roots (g)	2.03 ± 0.14 a	3.25 ± 0.16 b	2.80 ± 0.17 b	2.88 ± 0.15 b	1.47 ± 0.08 a	**<0.001**	RM
Wtotal (g)	4.90 ± 0.29 b	7.21 ± 0.28 d	6.05 ± 0.29 c	6.32 ± 0.28 cd	3.51 ± 0.16 a	**<0.001**	RM
RGR (mgg^{-1} day^{-1})	14 ± 0.8	13.5 ± 0.7	13.4 ± 0.7	12.9 ± 0.6	13.4 ± 0.7	n.s.	L
LA (cm^2)	146.0 ± 8.1 b	184.1 ± 7.7 c	142.7 ± 6.5 b	148.7 ± 6.5 b	98.7 ± 5.6 a	**<0.001**	RM
LAR (cm^2g^{-1})	31.1 ± 1.0 c	26.2 ± 0.8 ab	24.7 ± 0.7 a	24.0 ± 0.8 a	28.1 ± 0.9 bc	**<0.001**	RM
LWR (gg^{-1})	0.431 ± 0.009 b	0.379 ± 0.009 a	0.396 ± 0.010 ab	0.374 ± 0.011 a	0.418 ± 0.011 b	**<0.001**	RM
S/R (gg^{-1})	1.52 ± 0.06 b	1.34 ± 0.05 ab	1.30 ± 0.05 a	1.34 ± 0.06 ab	1.48 ± 0.07 ab	**<0.05**	RM
n	71	88	90	90	82		

Plant structure (leaf area ratio [LAR], leaf weight ratio [LWR], and shoot to root ratio [S/R]) showed a clear trend, with the highest values in populations located at the boundaries of the species distribution range (Table 5, Fig. 3). Leaf structure (SLA) also followed a similar pattern.

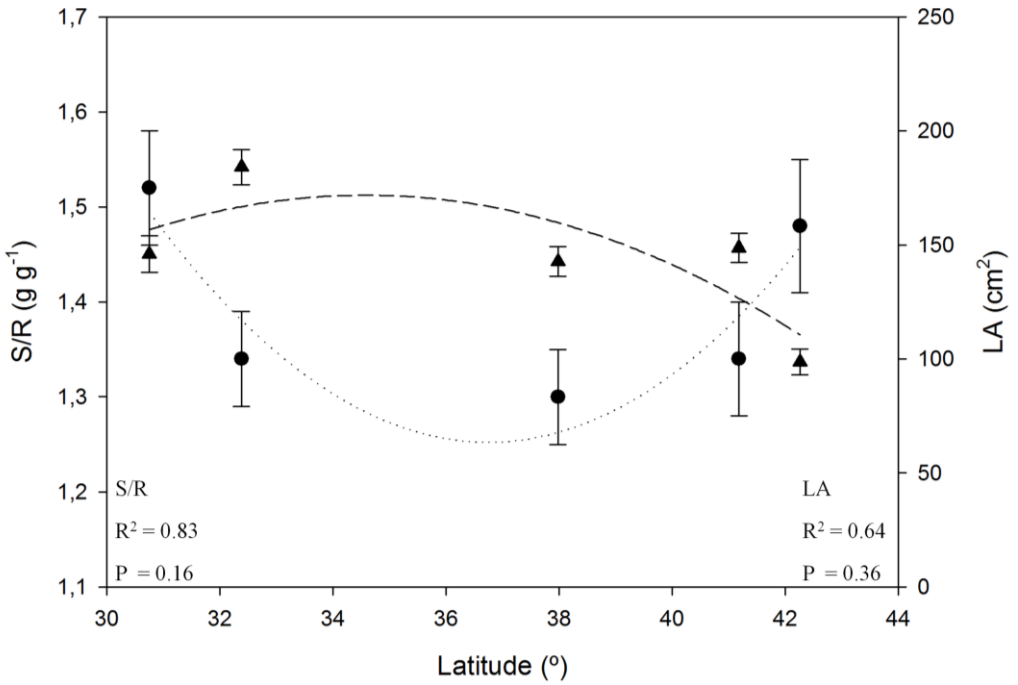

Figure 3. Relationships between latitude of origin and seedling biomass allocation (S/R) in *Quercus ilex* –left axis, circles– and seedling total leaf surface (LA) –right axis, triangles– for each population studied. Dotted line represents S/R trend with latitude and dashed line represent LA trend with latitude. Goodness of fit (R^2) and p-value (*P*) of quadratic regressions are given.

Finally, it is important to note that the populations exhibited significant inter-annual variability for relative growth rate (RGR) (Table 3), and also significant among population variability for acorn size, leaf traits and plant structure. The RGR did not differ significantly among populations but the seedling showed significant lower values in populations native to boundaries of the distribution range compared with core ones (Table 5, Fig. 5a).

4.4. Climate and population-trait models

The models selected on the basis of AICc to explain trait variance are summarized in Table 6 and detailed in Appendix S3. The model selected to explain the acorn fresh mass variance for *Q. ilex* – regarding climatic variables in each location for the four

years of study – accounted for 39% of the acorn mass variance (Fig. 4a and Table 6a). Both the mean minimum temperature (Tmmin_year) and solar radiation in April (Rapr) showed positive effects on acorn mass: years with a high mean minimum temperature and high solar radiation in April resulted in greater acorn mass. A significant linear relationship ($P < 0.05$) was noted between the random effect of the model (included as population) and aridity index values (de Martonne AI) (see Appendix S1 for an explanation) in native habitats for each population (Fig. 4e), as well as with the latitude (Fig. 4d).

For germination time (T_{50}) the model selected explained 76% of the variance (Fig. 4b and Table 6a). Acorn mass and mean temperature in May showed a negative effect on T_{50} (larger acorns together with years with high temperatures in May resulted in shorter germination time for the 50% of the acorns sown), whereas rainfall in January showed a positive effect in T_{50} (years with high rainfall in January resulted in a longer germination time). For the percentage germinated after 40 days (accumulated germination) the model selected explained the 62% of the variance (Fig. 4c and Table 6a), with a positive effect of both acorn mass and aridity (bigger acorns together with years with high aridity resulted in a higher accumulated germination percentage).

Table 6. Summary of linear mixed models selected to explain a) the effect of climate on acorn traits and b) the effect of seedling traits on seedling total weight and relative growth rate (RGR) for five *Quercus ilex* populations from Spain and Morocco. A null model considering tested variables as a constant was also tested. Models were selected on the basis of the Akaike information criterion corrected for small samples (AICc). n, number of samples; *K*, number of explaining variables plus one constant plus the error; *Wi*, relative probability of being the best model for the observed data. (+)/(–) denote positive/negative effect for variables.

	Model	Fixed effects	Random effect	n	*K*	*Wi*
a)	*Acorn mass*	Tmmin_year (+) Rapr (+)	Population	2408	4	0.94
	T_{50}	Acorn mass (−) P_jan (+) T_may (−)	Population	19	5	0.77
	Acumulated germination	Acorn mass (+) Aridity (+)	Population	19	4	0.77
b)	*Wtotal*	Acorn mass (+) LW (+) RGR (+)	Population	326	5	0.85
	RGR	Acorn mass (+) LW (+) LA (+)	Population	326	5	1.00

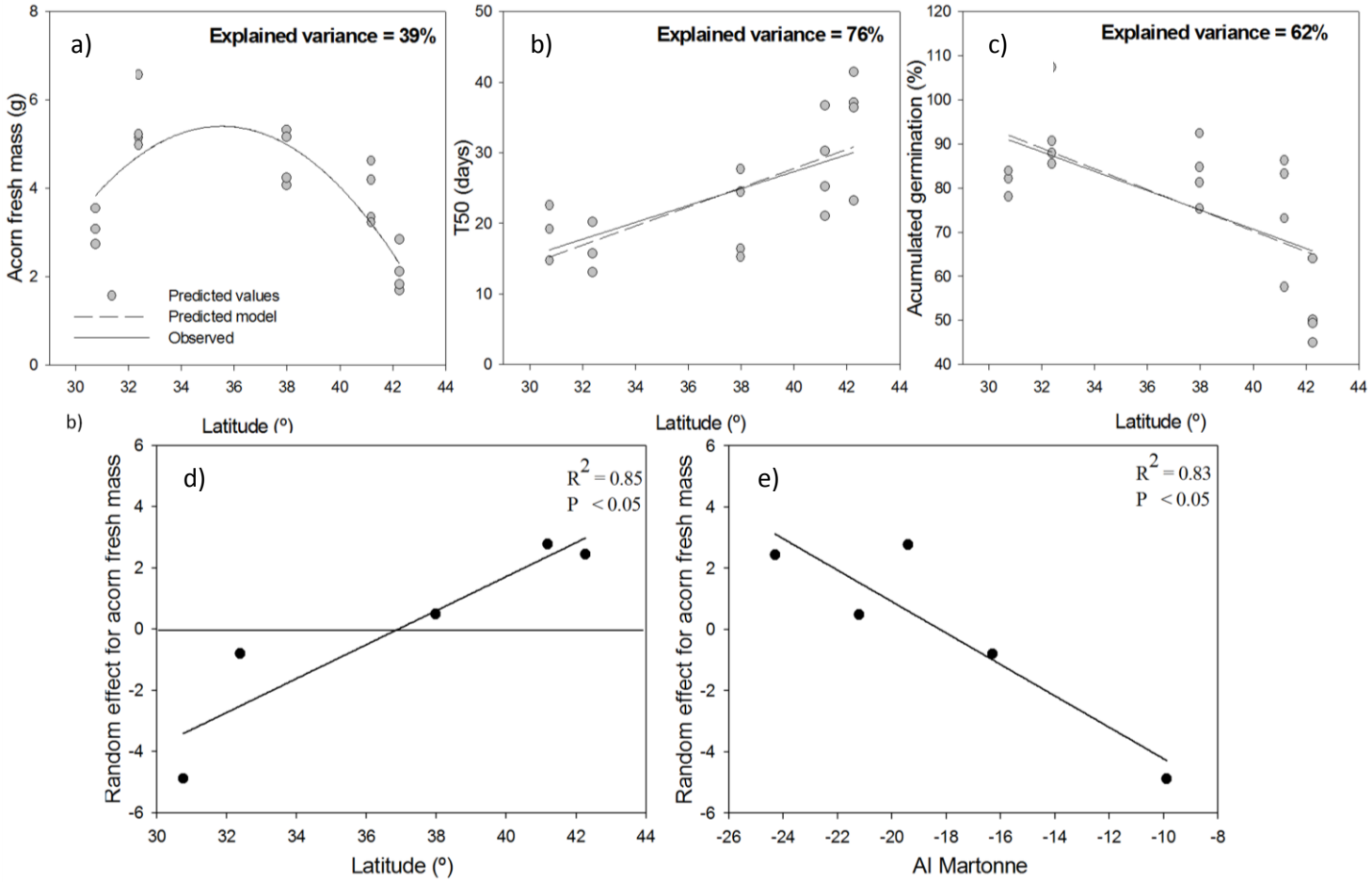

Figure 4. Relationships between latitude of origin and acorn fresh mass (a), T50 (b) and accumulated germination percentage (c) for the five *Quercus ilex* populations from Spain and Morocco in four years of study. Solid line represents the observed trend of the variable relative to latitude, dashed line represents the predicted trend by the selected model and grey circles represent predicted mean values for each year of study. Explained variance for each variable determined from the chosen model is displayed. Relationship between latitude and random effect for acorn fresh mass determined from the selected model (d), and relationship between such random effect and De Martonne Aridity Index (e).

The model selected to explain the variance in total weight for *Q. ilex* seedlings – based on morphological traits in four years of study – accounted for 77% of the total weight variance (Fig. 5a and Table 6b). Acorn mass, LW and RGR showed a positive effect on total seedling weight (seedlings from large acorns, with high individual leaf

weight, and high growth rate resulted in larger seedlings). The random effect of the model (included as population) showed lower values in populations native to the periphery of distribution range compared with core populations (Fig. 5c). The model selected for *Q. ilex* seedlings explained 56% of RGR variance (Fig. 5b and Table 6b). Acorn mass, LW and LA showed a positive effect in the growth rate (seedlings from large acorns, with high individual leaf weight and high total leaf area had faster growth rates). No model regarding morphological traits could be fitted for biomass allocation variables (Table S3.6 in Appendix S3).

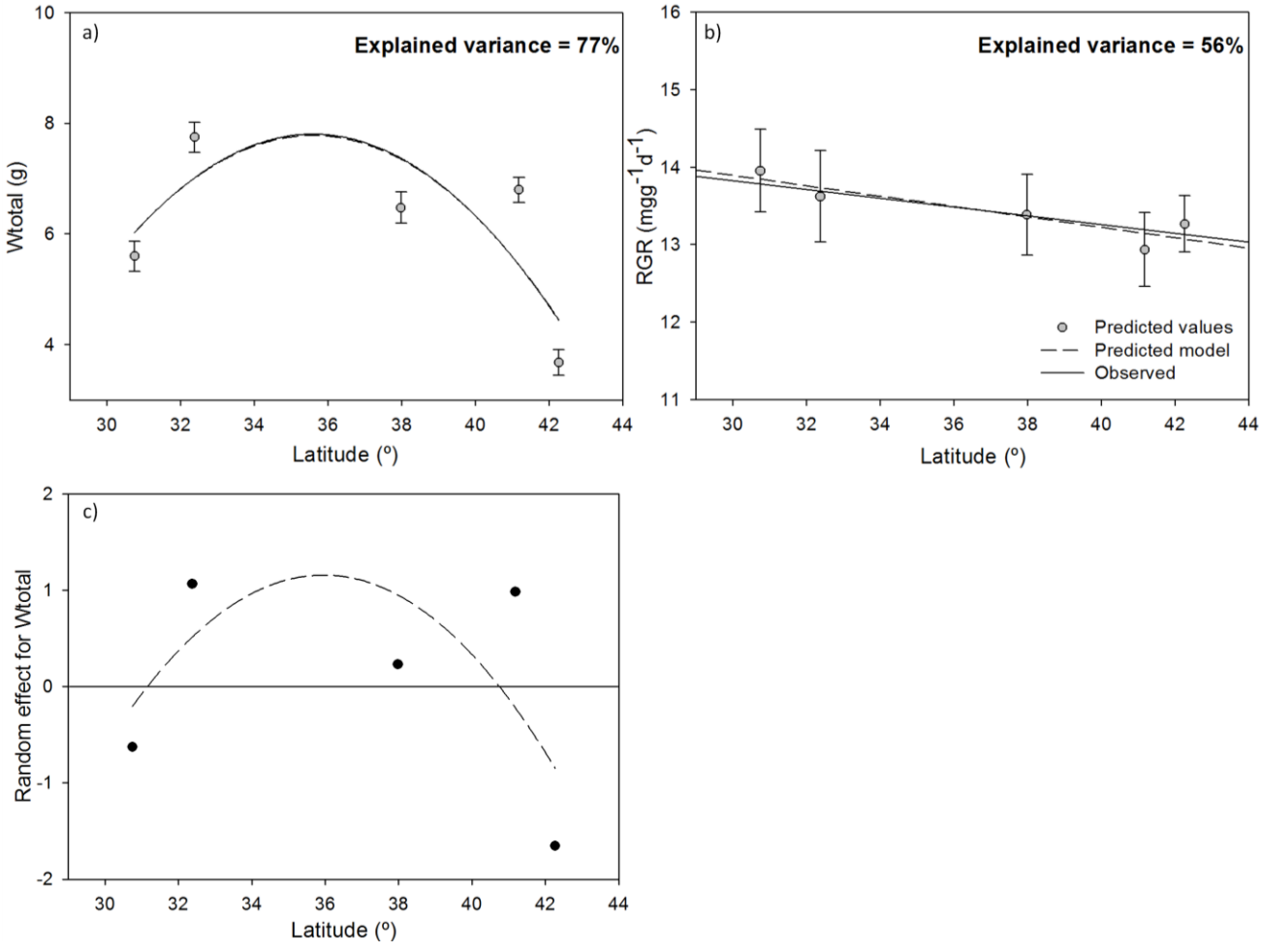

Figure 5. Relationship between latitude of origin and the seedlings total weight (a) and RGR (b) averaged of four years of study for five *Quercus ilex* populations from Spain and Morocco. The solid line represents the observed trend of the variable relative to latitude, dashed line represents the predicted trend by the selected model and grey circles represent predicted mean values for each year of study. Explained variance for each variable determined from the chosen model is displayed. Relationship between latitude and random effect for total seedling weight extracted from the selected model (c).

5. DISCUSSION

Environmental conditions at the range limits of the *Q. ilex* latitudinal distribution (low temperatures at northern edge and drought and warm temperatures at southern edge, Table 1) as well as lower foliar nitrogen concentrations (Table 2) at both range limits, reflect stressful growth conditions and may account for stress-induced convergent adaptive processes at both the northern and southern edges (Grime *et al*., 1997; Wright *et al*., 2004). Our results demonstrate several range margin patterns, for instance in tree and seedling size, acorn mass, and biomass allocation, according to core-edge provenances (Fig. 3, 4 and 5, and Tables 2, 4 and 5). Furthermore, these range margin patterns were verified for several years, despite a significant within-populations inter-annual variability (Table 3).

5.1. Range margin patterns. Phenotypic variation focused on edge populations

It is known that acorn mass of some *Quercus* species decreases with latitude (Koenig *et al*., 2009; Ramírez-Valiente *et al*., 2009); however, in contrast to our study, previous studies did not include both latitudinal range edges. Here we obtained spatial differences in acorn mass among populations, supporting the previously observed decrease. Notwithstanding this, contrary to previous findings, acorns from the southernmost population showed a smaller size than expected, resulting in a curve pattern (Fig. 4a). Therefore, the spatial variability registered for acorn mass provides a range margin pattern (i.e. similarities between edge versus core populations), instead of a latitude-related linear one; suggesting a relationship between stressful conditions at the range limits (low water availability at the southern limit, low temperature and radiation at the northern limit, Table 1) and reduced acorn mass (see also Aizen &

Woodcock, 1992). In addition, foliar nitrogen concentrations were lower at the range limits (Table 2), also suggesting stress-induced convergent traits in edge populations.

The variability in seedling morphology among the populations studied here is similar to that of other studies (García *et al.*, 1998; Gratani *et al.* 2003, Sánchez-Vilas & Retuerto 2007, Andivia *et al.* 2012), indicating high heterogeneity and suggesting local responses to environmental conditions at a population level for such traits. Mean seedling biomass was related mainly to the relative growth rate (49.6% of observed variance; Table S3.8 in Appendix S3), as well as to acorn mass and individual leaf weight (Table 6b; see also Ke & Werger, 1999; Baratolo *et al*., 2005; Quero *et al*., 2007; Bonito *et al*., 2011). In addition, seedling biomass also shows a range margin pattern, as lower mean seedling biomass was detected in the model as a random factor related to the range limits but unrelated to the fixed factors selected (Fig. 5c). The smaller seedling size found at the range limits, once the maternal effect of the acorn biomass, the RGR and the leaf weight effect were accounted for, agreed with the stress-resistance syndrome (Chapin *et al*., 1993). Thus, plants coming from stressful and unpredictable environments show constitutive lower growth, allocating a higher amount of energy to stress-resistance systems. Indeed, previous research has shown that evergreen oak populations from stressful environments (for instance gypsum soils) display constitutively higher maintenance costs, both in the laboratory as well as under field conditions (Laureano *et al*., 2008 and 2013), and therefore a lower energy budget available for growth.

Southwardly declining allocation to aboveground biomass (S/R, LWR and LAR) agrees with prior studies conducted in *Quercus* species (García *et al*., 1998; Bruschi, 2010), and may be related to adaptive responses to declining water availability and

higher radiation at lower latitudes (Long & Jones, 1996; Sánchez-Gómez *et al*., 2008; Sustani *et al*., 2014). Nonetheless this theoretical latitudinal trend was not validated in our study at the southernmost distribution limit, where biomass allocation values were similar to those found at the northern distribution limit, maintaining this surprising pattern (edge versus core populations) over the four study years (Fig. 3, Table 5 and Table S2.3. in Appendix S2). Furthermore, biomass-allocation patterns were not related to plant morphology or climatic variables (Tables S3.6 and S3.7 in Appendix S3). This result could be related to a short growth period at both the northern and southern distribution limits, by low temperatures and drought stress, respectively; promoting at both population edges, allocation to aerial biomass in order to maximize energy uptake during the limited period favourable to growth (Laureano *et al*., 2008 and 2013).

5.2. Latitudinal patterns in germination and leaf traits plasticity

Germination patterns showed high spatio-temporal variation, both among- and within-populations (Fig. 2, Tables 3 and 4). The positive effect of acorn mass on germination has previously been reported in *Quercus* (Gómez, 2004; Bonito *et al.*, 2011). Here, we demonstrate a significant effect of inter-annual climate variability, as well as an increasing germination speed at lower latitudes (Fig. 4b). It should be noted that May temperatures increase, while January rainfall decreases with decreasing latitude, thus resulting in a southward shortening of the time span available for the seedlings reach a viable size before the onset of drought (late May in NS and April in SM).

Leaf size (LS and LW) decreased with latitude, under both field and growth-chamber conditions (Tables 2 and 5), providing a constitutive pattern with reduced plasticity (Bruschi, 2010). Regarding SLA, despite the existence of a range margin trend

in the seedlings (Table 5), field data were not significantly different among populations. These results do not agree with previous studies (including *Quercus* species), which show both decreasing leaf size and SLA in water-limited populations (Bussotti *et al*., 2002; Bruschi *et al*., 2003; Marchin *et al*., 2008), as well as stressful nutritional conditions (Laureano *et al.*, 2013). A lack of clinal patterns in SLA may result from stochastic events (Anacker *et al*., 2011) or neutral evolution process (Ramírez-Valiente *et al*., 2011). Indeed, genetic data obtained from 162 markers provided by an AFLP analysis (See S.2.5. and Fig. S2.1. in Appendix S2) suggest a differentiation pattern following the northward colonization of Iberian Peninsula from North Africa (see Lumaret *et al*., 2002; Petit *et al*., 2005), supporting the contention of increasing genetic distance between northern and southern populations and suggesting that pattern of LS and LW may be related to neutral evolution process after colonization (Arend *et al*., 2011; Hatziskakis *et al*., 2011). Nonetheless it should be taken into account that such genetic analyses rely on a limited sample size, and reliable detection of genetic structure is potentially difficult with such a small sample size.

5.3. Temporal patterns. Accounting for inter-annual variability

Our results show that *Year* exerts strong effects on acorn mass and germination dynamics (Table 3), and such temporal variability is well explained by the annual climate fluctuations within population, as reflected the by GLMMs (Table 6a). On the other hand, the highly significant effect of *Population x Year* interaction obtained for acorn traits mainly resulted from the reduced seed quality of the northernmost population in 2011 (see S.2.6. and Fig S.2.2 in Appendix 2). With regards to seed mass, our data reveal both within- and among-population temporal patterns of variation, likely

related to adaptive capacity to cope with inter-annual environmental conditions (i.e. phenotypic plasticity), and long-term adaptation to average site conditions.

With regards inter-annual plasticity, years with warmer minimum temperatures and higher spring solar radiation are significantly related to increasing mean acorn mass, accounting for 39% of the observed variance (Table 6a and Table S3.8. in Appendix S3). Furthermore, regional climate seems to act as a selective pressure for acorn traits, as supported by the relationship between the random-factor coefficients (i.e. population scores) and mean site aridity (Fig. 4d-e). These results support the role of seed mass as a key functional trait, linked to plant adaptive capacity to stressful habitats (Marañón & Grubb, 1993, Fernández-Alés *et al.*, 1993; Alejano *et al.*, 2011). Contrary to our expectations, despite the common environmental conditions inside the growth chambers, substantial inter-annual variability within populations was also registered in seedling traits, likely as a result of maternal effects.

5.4. Concluding remarks

Extreme environmental conditions typical of the range limits seem to exert a convergent selective pressure toward stress resistance (García *et al.*, 1998; Hampe & Petit, 2005, Jump & Peñuelas, 2005). As a result, diversification and the appearance of similar population-level plant and leaf traits in *Q. ilex* might be associated with both northern and southern range edges of its distribution area, which are characterized by harsh environmental conditions.

Despite temporal patterns, a significant fraction of the phenotypic plasticity in plant morphology is linked to population provenance (Hoffmann & Parsons, 1997; Hoffmann & Hercus, 2000). Indeed, our results provide several range margin patterns

repeated for several years for seedlings grown under common environmental conditions. Thus, the among-population pattern for acorn mass, seedling size and biomass allocation (unrelated to inter-annual variability), upholds the idea of the existence of local fixed traits, likely with adaptive value.

Current climate change is producing range shifts of numerous taxa, communities and ecosystems worldwide. The consequences of such changes are typically most evident at the edge of the geographical distribution, where differences in growth or population dynamics may result in range expansions or contractions. Although it is widely accepted that a significant fraction of plant adaptive capacity relies on phenotypic plasticity, as an expression of diversity, quantifications of range-induced variation in life history phenotypes are still scarce. Our study reveals similar phenotypic traits among individuals native to the periphery of the distribution range, suggesting that the species phenotypic plasticity observed relies, to a large extent, on the selective pressure imposed by stressful conditions at range margins**.** We illustrate the critical importance of edge populations as long-term stores of species genetic resources, phenotypic plasticity and, as a result, adaptive capacity. The selective pressure of stressful environments at the range edges of plant species may be important for survival and evolution under a climate change scenario. Finally, we concur with Channell & Lomolino (2000) that conservation practices might gain significant insights by the understanding of ecological features and dynamics of range-limits populations.

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7. SUPPORTING INFORMATION

Appendix S1 Further information about study sites characterization

Extended explanation of the climatic-data calculation for the five *Q. ilex* populations

To analyse climatic trends at the study sites and also to quantify relationships of climate vs. individual traits, we used monthly climatic data (mean, maximum and minimum temperatures, and total precipitation) from the E-OBS v10.0 and CRU TS 3.22 datasets for the period 1950–2013 (KNMI Climate Explorer dataset: https://climexp.knmi.nl) (Oldenborgh & Burguers, 2005). These datasets correspond to interpolated data recorded by a dense network of local meteorological stations, which have been subjected to homogeneity tests and relative adjustments, and finally gridded onto a 0.25-0.5° network. In addition, Hobo U-12 data loggers (Onset, Pocasset, MA, USA) were set up at the study sites to record air temperature and relative humidity for the period 2012-2013 (from May to December and from January to October, respectively). For temperature, the climatic interpolation was corrected for each location through linear correlations using Hobo recorded data (all correlations between regional and Hobo data were highly significant).

Monthly surface solar radiation for each location was obtained from the FRESCO v6 dataset gridded at 0.5º for the period 2002-2012 (KNMI Climate Explorer).

Potential evapotranspiration (PET) was estimated as a function of the mean monthly temperatures, monthly rainfall and the geographical latitude (Thornthwaite 1948). Annual water balance was calculated as the difference between rainfall and potential evapotranspiration for each site. De Martonne aridity index was estimated as –

(Pa/(T+10)) (De Martonne 1926) –note that this aridity index has been calculated in negative values to give higher values of the index to locations with higher aridity–.

Table S1.1. Vegetation and soil characterization of the five *Q. ilex* study sites. The populations in the table are given in order of increasing latitude from left to right

	SM	**CM**	**SS**	**CS**	**NS**
Stand density (stands/ha)	736 ± 161	978 ± 71	513 ± 49	1115 ± 60	1165 ± 58
Site study area (ha)	22.0	42.2	10.1	11.7	11.1
Associated vegetation	*Chamaerops humilis, Ononis sp.*	*Chamaerops humilis, Cistus salviifolius, Pistacia lentiscus*	*Chamaerops humilis, Cistus ladanifer, Genista hirsuta, Quercus suber*	*Cistus ladanifer, Crataegus monogyna, Rosa sempervirens, Pinus pinaster*	*Cistus ladanifer, Daphne gnidium*
Soil type	Unstructured brown and eroded soil	Unstructured brown and eroded soil	Unstructured brown, siltstone soil	Unstructured brown, eroded soil	Unstructured brown, eroded soil
Bedrock	Limestone	Limestone	Slate rock	Colluvium	Colluvium
FAO classification (1991)	Lithosol	Lithosol	Cambisol	Lithosol	Lithosol

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Appendix S2 Supplementary results and genetic analyses

S2.1. Further statistical analyses to test differences for germination dynamics among populations and years.

In order to ascertain that parameters derived from sigmoidal functions matched the T_{50} and the accumulated germination percentage (x_o and a, respectively), we delimited groups of 100 acorns for population and year (pseudoreplication). Subsequently, we performed One-way ANOVA to test population effects.

The time to reach 50% germination (T_{50}) was calculated according to the following formula of Coolbear et al. (1984) modified by Farooq et al. (2005):

$$T50 = ti + [(N / 2 - ni) (ti - tj)] / (ni – nj)$$

Where, N is the final number of germinated acorns and ni, nj cumulative number of acorns germinated by adjacent counts at times ti and tj, respectively when ni < N / 2 < nj.

Table S2.1. Average and annual mean (±SE) values (2009, 2011, 2012, and 2013) of germination dynamic traits (T_{50} and accumulated germination percentage) calculated by pseudoreplication for each population. Different letters indicate statistical differences among locations in Tukey's HSD test. The populations in the table are given in order of increasing latitude from left to right

T_{50} (days)	**SM**	**CM**	**SS**	**CS**	**NS**	***P***
Average	**22.0 ± 0.6 ab**	**15.4 ± 0.8 a**	**21.1 ± 1.6 a**	**27.6 ± 1.4 bc**	**31.2 ± 2.2 c**	**<0.001**
2009	24.5 ± 0.8 c	12.0 ± 0.3 a	14.4 ± 0.6 a	38.0 ± 2.0 b	34.9 ± 1.7 b	<0.001
2011	19.5 ± 1.1 ab	19.3 ± 0.6 ab	16.6 ± 0.4 a	27.4 ± 1.1 bc	32.0 ± 4.5 c	<0.001
2012	-	10.8 ± 0.1 a	12.2 ± 0.2 b	19.4 ± 0.3 d	18.3 ± 0.4 c	<0.001
2013	22.0 ± 0.2 a	19.5 ± 0.5 a	34.5 ± 0.9 b	29.6 ± 1.6 b	41.2 ± 2.9 c	<0.001
Accumulated germination (%)	**SM**	**CM**	**SS**	**CS**	**NS**	*P*
Average	**81.9 ± 2.9 bc**	**91.5 ± 1.4 c**	**81.1 ± 2.8 c**	**67.7 ± 1.7 b**	**53.4 ± 5.5 a**	**<0.001**
2009	66.0 ± 4.0 b	97.0 ± 1.3 c	93.6 ± 1.5 c	72.8 ± 2.3 b	46.8 ± 2.3 a	<0.001
2011	81.4 ± 1.6 bc	79.2 ± 2.6 b	87.2 ± 1.8 c	78.0 ± 1.3 b	8.0 ± 0.2 a	<0.001
2012	-	95.5 ± 0.7 c	95.6 ± 1.0 c	67.8 ± 1.7 a	75.9 ± 1.4 b	<0.001
2013	92.3 ± 1.1 c	91.9 ± 0.5 c	61.3 ± 4.1 a	58.1 ± 2.0 a	74.5 ± 1.4 b	<0.001

S2.2. Study of year and population effect on acorn and seedling traits.

Table S2.2. Hierarchical analysis of variance partitioning the total variance (%) accounted by population and years within populations for morphological and phenological traits. Residual denotes the percentage of variance which is not absorbed by the main effects.

Effects	***Variance explained (%)***				
	Acorn mass	**LS**	**Leaves**	**Wtotal**	**LAR**
Population	36.2	29.7	16.4	22.2	3.3
Year	10.3	5.5	11.7	24.9	27.2
Residual	53.5	64.9	72.0	52.9	69.5
	T_{50}	**LW**	**Shoot**	**RGR**	**LWR**
Population	29.3	20.2	19.6	0.0	0.0
Year	55.5	5.5	12.3	1.2	28.0
Residual	15.2	74.3	68.1	98.8	72.0
	Accumulated germination	**SLA**	**Roots**	**LA**	**S/R**
Population	31.8	14.0	16.9	18.4	0.0
Year	62.2	8.4	35.4	9.1	14.9
Residual	6.0	77.6	47.8	72.5	85.1

S.2.3. Annual data for seedling traits

Table S2.3. Average and annual mean (±SE) values (2009, 2011, 2012, and 2013) of seedling traits for each population. Factorial ANOVA showed that both year and population significantly affected on seedling traits. Sample size is denoted in brackets. The populations in the table are given in order of increasing latitude from left to right

LS (cm^2)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**8.12 ± 0.29 (71)**	**7.75 ± 0.26 (88)**	**6.00 ± 0.18 (90)**	**6.01 ± 0.19 (89)**	**4.84 ± 0.15 (82)**
2009	7.96 ± 0.45 (37)	9.03 ± 0.41 (33)	6.25 ± 0.21 (35)	6.37 ± 0.36 (35)	4.89 ± 0.23 (35)
2011	8.74 ± 0.65 (14)	7.01 ± 0.51 (15)	6.41 ± 0.43 (15)	5.88 ± 0.35 (15)	4.28 ± 0.56 (8)
2012	-	7.64 ± 0.53 (20)	6.22 ± 0.52 (20)	5.53 ± 0.31 (20)	4.90 ± 0.26 (19)
2013	7.95 ± 0.45 (20)	6.29 ± 0.45 (20)	5.04 ± 0.36 (20)	5.97 ± 0.42 (19)	4.92 ± 0.26 (20)
LW (g)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**0.114 ± 0.004 (71)**	**0.113 ± 0.004 (88)**	**0.097 ± 0.003 (90)**	**0.094 ± 0.003 (89)**	**0.073 ± 0.002 (82)**
2009	0.109 ± 0.007 (37)	0.129 ± 0.006 (33)	0.099 ± 0.003 (35)	0.100 ± 0.006 (35)	0.072 ± 0.003 (35)
2011	0.125 ± 0.008 (14)	0.102 ± 0.008 (15)	0.105 ± 0.007 (15)	0.088 ± 0.006 (15)	0.059 ± 0.008 (8)
2012	-	0.113 ± 0.008 (20)	0.107 ± 0.010 (20)	0.087 ± 0.005 (20)	0.078 ± 0.005 (19)
2013	0.116 ± 0.007 (20)	0.097 ± 0.008 (20)	0.079 ± 0.006 (20)	0.097 ± 0.007 (19)	0.075 ± 0.004 (20)

SLA (cm^2 g^{-1})	**SM**	**CM**	**SS**	**CS**	**NS**
Average	71.8 ± 1.2 (71)	68.8 ± 0.9 (88)	62.4 ± 0.7 (90)	64.5 ± 0.6 (89)	66.9 ± 0.7 (82)
2009	74.5 ± 2.1 (37)	70.7 ± 1.5 (33)	63.7 ± 1.2 (35)	65.4 ± 1.0 (35)	68.1 ± 1.4 (35)
2011	67.7 ± 1.3 (14)	69.9 ± 2.9 (15)	61.7 ± 1.8 (15)	66.9 ± 1.1 (15)	73.0 ± 1.4 (8)
2012	-	68.1 ± 1.9 (20)	58.9 ± 1.1 (20)	64.5 ± 1.5 (20)	63.2 ± 1.1 (19)
2013	69.5 ± 1.5 (20)	65.7 ± 1.4 (20)	64.1 ± 1.0 (20)	61.1 ± 1.2 (19)	65.8 ± 1.1 (20)

Leaves (g)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**2.06 ± 0.12 (71)**	**2.63 ± 0.10 (88)**	**2.32 ± 0.11 (90)**	**2.29 ± 0.10 (90)**	**1.47 ± 0.08 (82)**
2009	1.64 ± 0.12 (36)	2.60 ± 0.16 (33)	2.24 ± 0.11 (35)	2.15 ± 0.13 (35)	1.54 ± 0.11 (35)
2011	2.94 ± 0.22 (15)	2.61 ± 0.17 (15)	2.86 ± 0.28 (15)	2.80 ± 0.25 (15)	0.81 ± 0.13 (8)
2012	-	2.96 ± 0.22 (20)	2.60 ± 0.28 (20)	1.98 ± 0.17 (20)	1.40 ± 0.19 (19)
2013	2.14 ± 0.24 (20)	2.37 ± 0.21 (20)	1.78 ± 0.25 (20)	2.48 ± 0.28 (20)	1.69 ± 0.13 (20)
Shoot (g)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**2.88 ± 0.17 (71)**	**3.96 ± 0.15 (88)**	**3.25 ± 0.16 (90)**	**3.43 ± 0.16 (90)**	**2.03 ± 0.10 (82)**
2009	2.15 ± 0.16 (36)	3.67 ± 0.24 (33)	2.99 ± 0.17 (35)	2.89 ± 0.16 (35)	2.01 ± 0.15 (35)
2011	3.98 ± 0.32 (15)	3.97 ± 0.28 (15)	4.07 ± 0.38 (15)	4.28 ± 0.42 (15)	1.09 ± 0.18 (8)
2012	-	4.47 ± 0.34 (20)	3.54 ± 0.39 (20)	2.99 ± 0.25 (20)	1.97 ± 0.24 (19)
2013	3.35 ± 0.36 (20)	3.90 ± 0.36 (20)	2.81 ± 0.41 (20)	4.20 ± 0.44 (20)	2.51 ± 0.18 (20)
Roots (g)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**2.03 ± 0.14 (71)**	**3.25 ± 0.16 (88)**	**2.80 ± 0.17 (90)**	**2.88 ± 0.15 (90)**	**1.47 ± 0.08 (82)**

2009	1.31 ± 0.08 (36)	2.57 ± 0.22 (33)	2.10 ± 0.13 (35)	1.96 ± 0.11 (35)	1.33 ± 0.08 (35)
2011	3.41 ± 0.36 (15)	4.38 ± 0.37 (15)	4.80 ± 0.57 (15)	4.48 ± 0.38 (15)	0.92 ± 0.12 (8)
2012	-	3.45 ± 0.31 (20)	2.48 ± 0.17 (20)	2.28 ± 0.22 (20)	1.37 ± 0.16 (19)
2013	2.27 ± 0.21 (20)	3.34 ± 0.31 (20)	2.83 ± 0.36 (20)	3.91 ± 0.25 (20)	2.04 ± 0.23 (20)

Total (g)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**4.90 ± 0.29 (71)**	**7.21 ± 0.28 (88)**	**6.05 ± 0.29 (90)**	**6.32 ± 0.28 (90)**	**3.51 ± 0.16 (82)**
2009	3.46 ± 0.23 (36)	6.25 ± 0.41 (33)	5.09 ± 0.28 (35)	4.84 ± 0.19 (35)	3.34 ± 0.20 (35)
2011	7.39 ± 0.61 (15)	8.35 ± 0.58 (15)	8.87 ± 0.81 (15)	8.76 ± 0.76 (15)	2.01 ± 0.28 (8)
2012	-	7.92 ± 0.59 (20)	6.01 ± 0.49 (20)	5.27 ± 0.42 (20)	3.34 ± 0.34 (19)
2013	5.63 ± 0.52 (20)	7.25 ± 0.64 (20)	5.65 ± 0.73 (20)	8.11 ± 0.61 (20)	4.55 ± 0.34 (20)

RGR (mgg-1day-1)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**14.0 ± 0.8 (52)**	**13.5 ± 0.7 (69)**	**13.4 ± 0.7 (71)**	**12.9 ± 0.6 (71)**	**13.4 ± 0.7 (54)**
2009	15.2 ± 1.5 (17)	14.4 ± 1.9 (14)	15.3 ± 1.5 (16)	12.2 ± 0.9 (16)	13.2 ± 1.6 (16)
2011	12.9 ± 1.2 (15)	12.0 ± 0.9 (15)	12.8 ± 1.3 (15)	12.3 ± 1.2 (15)	-
2012	-	13.7 ± 1.2 (20)	12.8 ± 1.2 (20)	13.0 ± 1.2 (20)	12.9 ± 1.5 (19)
2013	13.7 ± 1.4 (20)	13.9 ± 1.3 (20)	12.9 ± 1.8 (20)	14.0 ± 1.1 (20)	14.0 ± 1.2 (19)

LA (cm^2)	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**146.0 ± 8.1 (71)**	**184.1 ± 7.7 (88)**	**142.7 ± 6.5 (90)**	**148.7 ± 6.5 (89)**	**98.7 ± 5.6 (82)**
2009	123.0 ± 8.9 (37)	185.2 ± 12.5 (33)	141.2 ± 7.1 (35)	141.3 ± 8.4 (35)	106.5 ± 8.3 (35)
2011	202.7 ± 17.4 (14)	196.9 ± 19.5 (15)	174.6 ± 17.3 (15)	186.7 ± 16.8 (15)	58.8 ± 9.6 (8)
2012	-	201.7 ± 16.4 (20)	151.9 ± 15.4 (20)	126.3 ± 10.4 (20)	87.9 ± 11.4 (19)
2013	147.6 ± 16.3 (20)	155.1 ± 14.8 (20)	112.1 ± 15.4 (20)	155.6 ± 18.1 (19)	111.1 ± 9.0 (20)

LAR (cm^2g^{-1})	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**31.1 ± 1.0 (71)**	**26.2 ± 0.8 (88)**	**24.7 ± 0.7 (90)**	**24.0 ± 0.8 (89)**	**28.1 ± 0.9 (82)**
2009	35.3 ± 1.5 (37)	30.6 ± 1.5 (33)	28.5 ± 0.9 (35)	29.0 ± 1.3 (35)	31.1 ± 1.2 (35)
2011	27.4 ± 0.9 (14)	23.6 ± 1.4 (15)	20.5 ± 1.6 (15)	21.3 ± 1.0 (15)	27.1 ± 0.5 (8)
2012	-	25.5 ± 1.0 (20)	25.0 ± 1.1 (20)	24.5 ± 1.2 (20)	26.2 ± 2.0 (19)
2013	26.2 ± 1.2 (20)	21.7 ± 1.0 (20)	21.0 ± 1.4 (20)	17.0 ± 1.5 (19)	25.0 ± 1.3 (20)

LWR (gg^{-1})	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**0.431 ± 0.009 (71)**	**0.379 ± 0.009 (88)**	**0.396 ± 0.010 (90)**	**0.374 ± 0.011 (89)**	**0.418 ± 0.011 (82)**
2009	0.473 ± 0.010 (37)	0.428 ± 0.015 (33)	0.446 ± 0.010 (35)	0.441 ± 0.017 (35)	0.453 ± 0.012 (35)
2011	0.406 ± 0.015 (14)	0.337 ± 0.015 (15)	0.329 ± 0.022 (15)	0.318 ± 0.014 (15)	0.372 ± 0.008 (8)
2012	-	0.379 ± 0.017 (20)	0.424 ± 0.018 (20)	0.378 ± 0.013 (20)	0.412 ± 0.030 (19)
2013	0.377 ± 0.015 (20)	0.331 ± 0.016 (20)	0.328 ± 0.021 (20)	0.294 ± 0.018 (19)	0.382 ± 0.021 (20)

S/R (gg^{-1})	**SM**	**CM**	**SS**	**CS**	**NS**
Average	**1.52 ± 0.06 (71)**	**1.34 ± 0.05 (88)**	**1.30 ± 0.05 (90)**	**1.34 ± 0.06 (90)**	**1.48 ± 0.07 (82)**
2009	1.63 ± 0.08 (36)	1.58 ± 0.09 (33)	1.47 ± 0.07 (35)	1.60 ± 0.11 (35)	1.56 ± 0.09 (35)
2011	1.26 ± 0.11 (15)	0.95 ± 0.06 (15)	0.93 ± 0.10 (15)	0.96 ± 0.06 (15)	1.18 ± 0.11 (8)
2012	-	1.37 ± 0.09 (20)	1.48 ± 0.14 (20)	1.44 ± 0.13 (20)	1.57 ± 0.18 (19)
2013	1.52 ± 0.11 (20)	1.22 ± 0.09 (20)	1.10 ± 0.09 (20)	1.08 ± 0.10 (20)	1.39 ± 0.12 (20)

S.2.4. Acorn fresh mass range and number of acorns considered for germination

Table S2.4. Acorn fresh mass range per population for each experimental year. These acorns were the remaining samples after subset the acorns taken for dry weight and the discarding of infected ones. The size sample is denoted in brackets. The populations in the table are given in order of increasing latitude from left to right

Acorns mass (g)	SM	CM	SS	CS	NS
2009	0.87-12.06 (501)	2.03-14.36 (501)	1.27-10.04 (501)	1.36-8.65 (502)	0.86-5.48 (521)
2011	1.13-7.91 (500)	1.22-11.92 (500)	1.47-12.89 (990)	1.15-11.72 (500)	0.26-3.43 (376)
2012	-	0.55-11.22 (1000)	1.18-11.14 (996)	0.46-7.84 (954)	0.46-5.67 (1000)
2013	0.40-7.48 (833)	0.72-14.59 (829)	0.80-15.00 (1194)	0.34-9.20 (795)	0.32-5.37 (888)

S2.5. Genetic analyses

A preliminary genetic analysis was performed in order to assess the genetic structure of the study populations. Fresh leaf material was collected from each population (or 5 trees per population) and dried at ambient temperature. Total genomic DNA was extracted from approximately 35 mg of ground leaf material using DNeasy Plant Mini Kit (QIAGEN) and the manufacturer protocol. The AFLP analysis was performed as originally described by Vos *et al.* (1995), with some modifications described in Herrera & Bazaga (2009). We used three *Eco*RI + 3/ *Mse*I primer combinations, chosen from a broader sample of combinations previously assayed in a pilot study. Fragment separation and detection was made using an ABI PRISM 3100 DNA sequencer. The presence or absence of each marker in each individual plant was scored manually by visualizing electrophoregrams with GeneMapper 3.7 sofware. All scoring was carried out by the same person, who during the process lacked any information on population location. From the combination of the restriction enzymes we obtained 162 markers (loci) in the size range 150-500 bp.

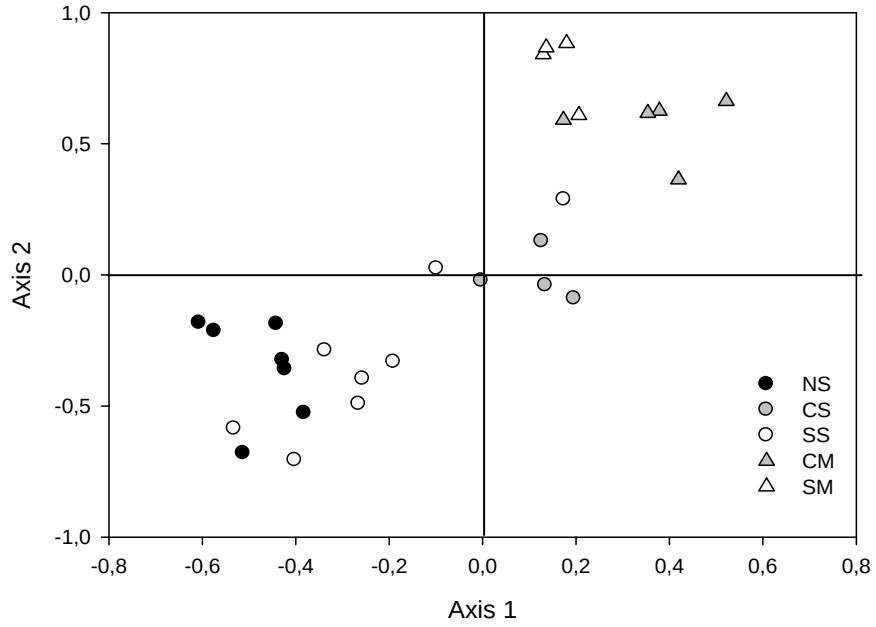
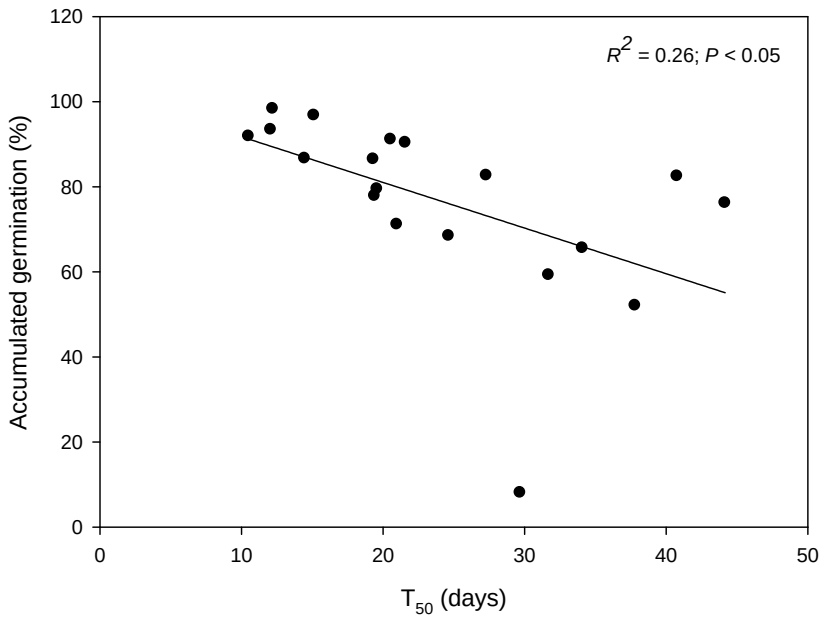


Figure S2.1. Principal Components Analysis plot based on genetic similarities of AFLP data of the five *Q. ilex* populations considered (absorbed variance first axis = 23.9%, second axis = 22%). Population codes: NS: northern Spain, CS: central Spain, SS: southern Spain, CM: central Morocco, SM: southern Morocco.

S.2.6. Likelihood of extreme events and seed germination rate.

Our study suggests that the likelihood of extreme events is higher at the distribution limits. The northern population in 2011 registered an extremely low germination rate due to "drippy nut" possibly caused by a bacterial infection (Koenig et al., 2013), while the southernmost population showed extremely low acorn production in 2012. The correlation found between cumulative germination and germination time (Fig. S2.2. in Appendix S2) could be explained by the fact that fast germination improves seed viability (Connor & Sowa, 2003). Besides, with respect to the extreme event probability, it has been reported that the frequency of climatic disturbance events is roughly twofold higher in the north of the distribution range of Q. ilex than in the south (García Herrera et al., 2001); therefore, we suggest that the increasing germination time may be favoured as a buffering mechanism to reduce the likelihood of random negative effects (Quintana et al., 2004; Verdú & Traveset, 2005).

Figure S2.2. Relationship between T_{50} and percentage of accumulated acorns germinated of the five populations in the four years of study. Note that the lowest value of accumulated germination corresponds to the NS population acorns harvested in 2011 ("drippy nut" event).



S2.7. Testing if acorn and seedling traits are useful to distinguish differences among populations.

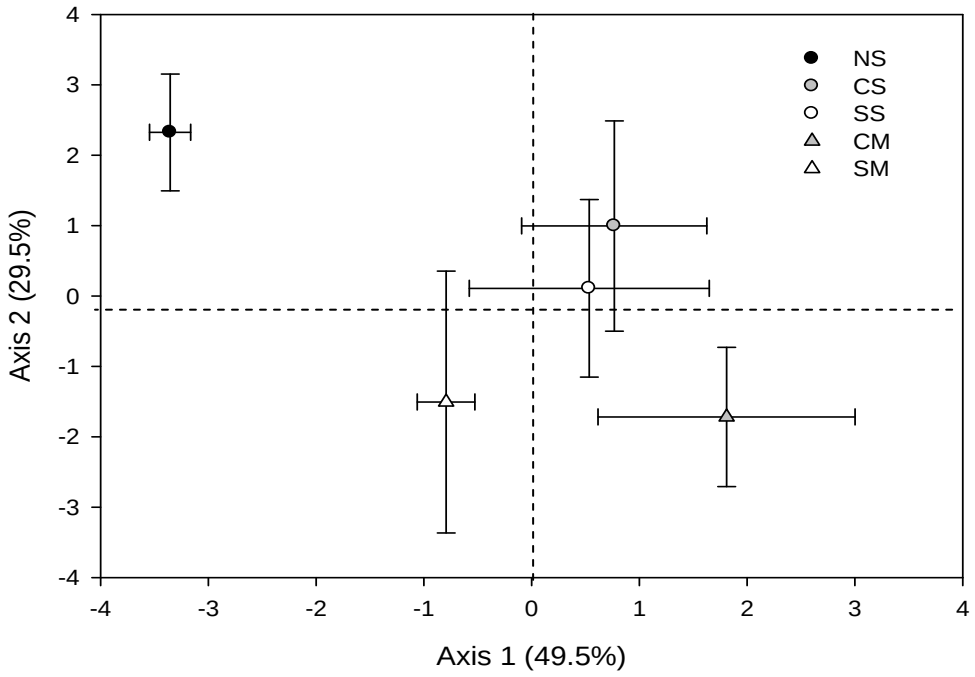


Figure S.2.3. PCA accounting the mean acorn and seedling traits of the five *Q. ilex* study sites for every year (n = 19).

Table S.2.5. Results of the PCA for morphological and acorn traits. In bold are evidenced the loading over 0.6. The principal factors represent the three factors with higher eigenvalues.

	Factor 1	***Factor 2***
T_{50}	-0.39	**0.60**
Accumulated	0.32	**-0.73**
Fresh acorn	0.41	**-0.64**
SLA	-0.23	-0.52
LS	0.15	**-0.90**
LW	0.23	**-0.89**
LA	**0.83**	-0.47
LAR	**-0.74**	**-0.63**
LWR	**-0.78**	-0.56
RGR	**-0.70**	-0.45
Leaves	**0.88**	-0.38
Shoot	**0.96**	-0.17
Roots	**0.96**	0.12
S/R	**-0.78**	-0.45
Total	**0.99**	0.00

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Appendix S3 Detailed linear mixed models built to explain the effect of climate or morphological traits for the variables studied and detailed regression coefficients of the final full model selected for each variable considered. n, number of samples; *K*, number of explaining variables plus one constant plus the error; AIC, Akaike information criterion; AICc, Akaike information criterion corrected for small samples; Δi, difference in AICc with respect to the best model; *L(gi/x)*, probability of each model as being the actual K-L (discrepancy information value) best model; *Wi*, relative probability of being the best model for the observed data. (+)/(-) denote positive/negative effect for variables.

Table S3.1. Acorn mass

Model	Fixed effects	Random effect	n	*K*	AIC	AICc	Δi	*L(gi/x)*	*Wi*
1	**Tmmin_year + Rapr**	**Population**	**2408**	4	9156	**9156**	**0.00**	**1.00**	**0.94**
2	Tmin − Pjun + Rapr			5	9161	9161	5.4	0.07	0.06
3	Rad_apr			3	9210	9210	54.5	0.00	0.00
4	Tmmin_year			3	9247	9247	91.3	0.00	0.00
5	Tm_year			3	9256	9256	100.5	0.00	0.00
6	Pjun			3	9279	9279	123.5	0.00	0.00
7	Tmmax_year			3	9319	9319	163.6	0.00	0.00
8	− P_sep			3	9331	9331	175.9	0.00	0.00
9	− P_spring			3	9355	9355	199.7	0.00	0.00
10	null model			2	9360	9360	204.2	0.00	0.00
11	− Tm_sep			3	9363	9363	207.4	0.00	0.00
12	Rad_year			3	9368	9368	212.5	0.00	0.00
13	Aridity			3	9369	9369	213.1	0.00	0.00
14	P_year			3	9371	9371	215.0	0.00	0.00

Table S3.2. T_{50}

Model	Fixed effects	Random effect	n	*K*	AIC	AICc	Δi	*L(gi/x)*	*Wi*
1	**− Acorn mass + P_jan − T_may**	**Population**	**19**	**5**	**126**	**130**	**0.00**	**1.00**	**0.77**
2	T_may			3	132	134	3.78	0.15	0.12
3	P_jan			3	134	136	5.48	0.06	0.05
4	Acorn mass			3	136	138	7.78	0.02	0.02
5	Tmmax_year			3	137	139	8.48	0.01	0.01
5	Tm_year			3	137	139	8.68	0.01	0.01
6	Aridity			3	137	139	8.78	0.01	0.01
7	Tmmin_year			3	139	140	10.08	0.01	0.01
8	P_su			3	140	142	11.48	0.00	0.00
9	null model			2	140	141	10.93	0.00	0.00
10	Rad_year			3	142	144	13.78	0.00	0.00
11	P_fa			3	146	148	17.38	0.00	0.00
12	P_wi			3	147	149	18.78	0.00	0.00
13	P_sp			3	148	149	18.88	0.00	0.00

Table S3.3. Accumulated germination percentage

Model	Fixed effects	Random effect	n	*K*	AIC	AICc	Δi	*L(gi/x)*	*Wi*
1	**Acorn mass + Aridity**	**Population**	**19**	**4**	**152**	**156**	**0.00**	**1.00**	**0.47**
2	Acorn mass			3	156	157	1.18	0.55	0.26
3	Tm_year + Acorn mass			4	155	158	1.34	0.51	0.24
4	T_jun			3	163	164	7.98	0.02	0.01
5	Tmmax_year			3	164	166	9.28	0.01	0.00
6	Tm_year			3	165	166	9.98	0.01	0.00
7	Aridity			3	165	167	10.48	0.01	0.00
8	P_aug			3	166	167	11.18	0.00	0.00
9	Tmmin_year			3	166	168	11.48	0.00	0.00
10	null model			2	168	169	12.33	0.00	0.00
11	P_su			3	167	169	12.68	0.00	0.00
12	Rad_year			3	169	171	14.58	0.00	0.00
13	P_wi			3	172	174	17.48	0.00	0.00
14	P_sp			3	172	174	17.78	0.00	0.00
15	P_fa			3	173	174	17.88	0.00	0.00
16	P_year			3	173	174	18.18	0.00	0.00

Table S3.4. Wtotal

Model	Fixed effects	Random effect	n	*K*	AIC	AICc	Δi	*L(gi/x)*	*Wi*
1	**Acorn mass + LW + RGR**	**Population**	**326**	**5**	**1168**	**1168**	**0.00**	**1.00**	**0.85**
2	Acorn mass – S/R − SLA + LS − LW + RGR			8	1171	1171	3.41	0.18	0.15
3	RGR			3	1213	1213	44.5	0.00	0.00
4	Acorn mass			3	1442	1442	274.3	0.00	0.00
5	LW			3	1463	1463	294.7	0.00	0.00
6	LS			3	1475	1475	307.2	0.00	0.00
7	− LWR			3	1531	1531	362.9	0.00	0.00
8	− LAR			3	1537	1537	369.0	0.00	0.00
9	null model			2	1540	1540	372.3	0.00	0.00
10	− S/R			3	1541	1541	373.4	0.00	0.00
11	− SLA			3	1545	1545	377.3	0.00	0.00

Table S3.5. RGR

Model	Fixed effects	Random effect	n	*K*	AIC	AICc	Δi	*L(gi/x)*	*Wi*
1	**Acorn mass + LW + LA**	**Population**	**326**	**5**	**1813**	**1813**	**0.00**	**1.00**	**1.00**
2	LA			3	1854	1854	40.8	0.00	0.00
3	LW			3	1992	1992	179.3	0.00	0.00
4	Acorn mass			3	1996	1996	183.0	0.00	0.00
5	LS			3	2005	2005	191.6	0.00	0.00
6	− LWR			3	2044	2044	230.9	0.00	0.00
7	S/R			3	2045	2045	231.9	0.00	0.00
8	null model			2	2046	2046	233.2	0.00	0.00
9	− LAR			3	2053	2053	239.5	0.00	0.00
10	− SLA			3	2053	2053	239.6	0.00	0.00

Table S3.6. S/R

Model	**Fixed effects**	**Random effect**	**n**	***K***	**AIC**	**AICc**	**Δi**	***L(gi/x)***	***W*i**
1	Acorn mass	Population	326	3	504	504	0.00	1.00	0.60
2	LW			3	506	504	2.14	0.34	0.20
3	null model			2	506	506.14	2.40	0.30	0.18
4	Wtotal			3	513	512.77	9.04	0.01	0.00
5	SLA			3	513	513.07	9.34	0.01	0.00
6	LS			3	513	513.37	9.64	0.01	0.00
7	RGR			3	514	514.57	10.84	0.00	0.00

Table S3.7. S/R *vs*. climate

Model	**Fixed effects**	**Random effect**	**n**	***K***	**AIC**	**AICc**	**Δi**	***L(gi/x)***	***Wi***
1	null model	Population	19	2	12.1	12.9	0.00	1.00	0.90
2	Fresh acorn			3	18.4	20.0	7.14	0.03	0.03
3	Pmay			2	19.8	21.4	8.54	0.01	0.01
4	Tmin			3	20.4	22.0	9.14	0.01	0.01
5	aridity			3	20.5	22.1	9.24	0.01	0.01
6	Tm			3	20.5	22.1	9.24	0.01	0.01
7	PETapr			3	20.6	22.2	9.32	0.01	0.01
8	Pdec			3	20.7	22.3	9.44	0.01	0.01
9	Tmax			3	20.8	22.4	9.54	0.01	0.01
10	Rjul			3	19.9	22.6	9.71	0.01	0.01
11	Pspring			3	21.5	23.1	10.24	0.01	0.01
12	Rapr			3	22.5	24.1	11.24	0.00	0.00

Table S3.8. Regression coefficients of the final full model selected explaining acorn and seedling traits. For each variable, the relative weight in the model (RWM), the variance explained (VE) and the total variance explained (Total VE) are indicated.

Variables	Fixed effects	Value	Std. Error	p-value	RWM (%)	VE (%)	Total VE (%)
Acorn mass	Tmmin_year	0.5921	0.0736	<0.001	53.2	20.7	39
	Rapr	0.0079	0.0008	<0.001	46.8	18.3	
T50	Acorn mass	-1.7475	1.0173	0.114	19.3	14.7	76
	P_jan	0.2599	0.0714	0.012	25.5	19.4	
	T_may	-1.0647	0.3831	0.004	55.2	41.9	
Accumulated germination	Acorn mass	9.9670	2.2989	0.001	59.1	36.7	62
	Aridity	1.6766	0.7049	0.035	40.9	25.3	
Wtotal	Acorn mass	0.2820	0.0516	<0.001	19.3	14.9	77
	LW	10.7013	2.8326	<0.001	16.3	12.5	
	RGR	0.3021	0.0160	<0.001	64.4	49.6	
RGR	Acorn mass	0.6793	0.1342	<0.001	23.1	12.9	56
	LW	23.4288	7.5956	0.002	17.7	9.9	
	LA	0.0504	0.0035	<0.001	59.2	33.2	

III.CAPÍTULO 2

Phenotypic variation of physiological and morphological traits relies on the tradeoff between stress tolerance and photosynthetic efficiency.

Phenotypic variation of physiological and morphological traits relies on the tradeoff between stress tolerance and photosynthetic efficiency.

Ana García-Nogales, Juan C. Linares, Rocío Cortés-Gañán, José I. Seco, José Merino

1. ABSTRACT

Intraspecific variability has become a central issue in the current research on plant adaptive capacity to global change. However, the variability across the whole range of distribution of plant species in relation to key plant functional traits is still poorly understood. Here, the phenotypic variation of photosynthetic and morphological traits was analyzed under controlled growth conditions in seedlings from 12 populations of the Mediterranean oak *Quercus ilex*. Phenotypic differentiation was significant among populations, while maximum PSII photochemical efficiency (F_v/F_m) lacked inter-population differentiation. For some traits, intraspecific variability was partially related to local climate; whereas variables related to photosynthetic efficiency (*PN*, Φ_{PSII}, and q_P), leaf size and structural traits (S/R, LAR) displayed quadratic trends with latitude, where the edge populations registered similar values. We conclude that harsh abiotic conditions at the distribution-range limits might exert a selective pressure to favor individuals with a phenotypic canalization strategy in terms of low photosynthetic efficiency. Although this strategy seems to limit the edge populations' capacity to use resources efficiently under non-limiting conditions, this is compensated through the structural traits. Our study underlines the importance of take into consideration population differentiation in key functional traits in order to predict the future species' distribution shifts.

Key-words: evergreen oak; latitudinal gradient; local adaptation; phenotypic canalization; phenotypic plasticity; photochemical quenching; photosynthetic rate; PSII photochemical efficiency; range edges; structural traits.

2. INTRODUCTION

Plant adaptive capacity to contrasting environmental conditions relies mainly on genetic diversity and phenotypic plasticity (Pigliucci 2006; Bresson *et al.* 2011). Nevertheless, the relative importance of such types of variation depends on both the trait studied and the environmental variable considered (Baquedano *et al.* 2008). Plastic responses appear to be as important as genetically based evolutionary potential to cope with rapid adaptive processes to changing climate (Hoffmann & Sgrò 2011). Therefore, wondering about population persistence under a climate change scenario, the quantification of the relative importance of either genetically based variation or phenotypic plasticity in populations native to contrasting environments seems crucial to grasp mechanisms linked to adaptation to environmental changes (Valladares *et al.* 2002; Vitasse *et al.* 2010).

Wide latitudinal and altitudinal gradients provide an experimental framework to investigate the intraspecific variability of particular plant functional traits, as well as to understand the implications of this variation to face changing environmental conditions (Oleksyn *et al.* 1998; Benowicz *et al.* 2000; Körner 2007; Vitasse *et al.* 2010; Linares *et al.* 2012). In such gradients, intraspecific differences in functional traits may be detected

and are likely related to local adaptations to abiotic conditions (for instance, temperature and water availability). Edge populations, living close to their physiological limits, provide valuable information about the adaptive capacity to stressful conditions and expected future changes in the species distribution ranges (Hoffmann & Sgrò 2011). Nonetheless, the study of the intraspecific variation of functional traits rarely corresponds to the entire biogeographical species range (but see Niinemets 2015; Reich *et al.* 2014).

The notion that strong relationships exist between the biogeographical location of populations and their stress resistance is becoming more accepted (Parsons 1993; García *et al.* 1998; Channell & Lomolino 2000). Overall, differential patterns found among populations of a given species should reflect biogeographical processes, since, at least from a physical standpoint, the core of the geographical range should present habitats more favorable to the species' physiology, while the range edges would represent the highest level of abiotic stress (Hampe & Petit 2005). Furthermore, border populations are commonly subjected to fluctuating environmental conditions where the likelihood of extreme events might be higher (Brown *et al.* 1996; Hampe & Petit 2005; Sexton *et al.* 2009). In summary, the stressful conditions of range edges may result in the selection of genotypes differentiated from the rest of populations native to less stressful environments (Bruschi 2010).

The study of individuals native to different geographical origins, but cultivated under homogeneous conditions, would allow the detection of constitutive differences in particular traits driven by environmental selective pressure, according to the habitat where the populations have evolved (Ramírez-Valiente *et al.* 2010). For instance, regarding photosynthetic characteristics related to gas exchange (photosynthetic rate,

stomatal aperture, water use efficiency, etc.) and/or to energy absorption and transport mechanisms (actual and maximum Photosystem II efficiency, electron transport rate, etc.), the occurrence of photosynthetic ecotypes would be expected (García-Plazaola *et al.* 2000). Typically, populations native to stressful environments display lower photosynthetic capacity because their activity is constrained either by nutrient limitation, extreme temperatures or water shortage (Poorter *et al.* 1991; Wright *et al.* 2006) as well as by the higher allocation of cellular resources to metabolic machinery related to stress defense and repair (Takashima *et al.* 2004; Laureano *et al*. 2016).

Holm oak (*Quercus ilex* L.) is an evergreen tree widely distributed throughout the Mediterranean Basin. *Q. ilex* shows specific adaptations to the Mediterranean climate (drought and high temperatures in summer, and low temperatures in winter), such as low specific leaf area (SLA) and strong control of stomatal aperture. This usually results in low values of photosynthetic rate and high water use efficiency (Gratani *et al.* 2013; Flexas *et al.* 2014). Owing to its broad distribution range, *Q. ilex* is adapted to contrasting water availability and temperature conditions. Evidence of genetic variability within *Q. ilex* species, specifically among populations, has come from prior studies reporting on morphological and physiological traits related to environmental conditions at the site of origin (García *et al.* 1998; Bonito *et al.* 2011; Laureano *et al.* 2013; García-Nogales *et al.* 2016; Varone *et al.* 2016). Here, we survey constitutive differences in functional traits related to the photosynthetic apparatus and morphology in *Q. ilex* subsp. *ballota* (Desf.) Samp. along a 1,800-km latitudinal gradient. Furthermore, we attempt to elucidate the underlying relationships between such functional traits and climate. We hypothesize that *Q. ilex* seedlings, native to populations inhabiting contrasting environments, growing under common non-limiting

conditions, will show constitutive differences in functional traits related to both the photosynthetic apparatus and the seedling morphology. Specifically, we hypothesize that populations native to the edges of the distribution area, characterized by stressful abiotic environments, will display range margin patterns for particular traits (i.e. differences between core *vs.* edge populations). The specific questions are:

1. Do the study populations exhibit constitutive differences in physiological and morphological traits?
2. If so, are these differences linked to climatic characteristics at the site of origin and/or to biogeography (core *vs.* periphery patterns)?

3. MATERIAL AND METHODS

3.1. Sampling areas and climate

We selected 12 populations of *Q. ilex* subsp. *ballota*, located along a latitudinal gradient covering a variety of climatic conditions throughout the distribution range of the subspecies (Table 1). Populations native to the southernmost and the northernmost latitudinal limits of the distribution range were included (see Fig. S1 in Appendix S1 in Supporting Information). The soil types ranged from shallow lithosols on slopes to deeper soils on flatter areas. The parent material was mainly calcareous in the eastern Iberian Peninsula and Morocco and metamorphic siltstone in the western Iberian Peninsula.

Table 1 describes the climatic characteristics of the 12 locations (for further details see Methods S1 in Appendix S3). The maximum mean summer temperature range from 33.5ºC in Agadir (the southernmost population) to 20.6 ºC in Cambo (the

northernmost population) and the minimum mean winter temperature range -0.5ºC in León (a northern population) to 10.6ºC in Agadir. The southernmost population (Agadir) was characterized by the maximum values of mean annual temperature, radiation, potential evapotranspiration, and aridity index; while the northernmost population (Cambo) was characterized by both the lowest aridity and the lowest maximum temperature over the year (Tables 1 and S2 in Appendix S2).

Table 1. Location and climatic characteristics of the 12 populations of *Q. ilex* ordered by latitude of origin (from north to south). Code, letters linked to locations. T, mean annual temperature; Tmax, mean maximum temperature in summer; Tmin, mean minimum temperature in winter; Pa, annual rainfall; PET, corrected potential evapotranspiration; De Martonne's aridity index, -(Pa/(T+10)); Rad, mean solar radiation; Thermal oscillation, difference between the mean maximum temperature in summer and the mean minimum temperature in winter.

Note: The De Martonne aridity index decreases as aridity increases; thus, we used negative values of the aridity index to avoid confusion.

Code	***Population***	***Country***	***Latitude (N)***	***Longitude (E-W)***	***Elevation (m.a.s.l.)***	***T (ºC)***	***Tmax (ºC)***	***Tmin (ºC)***	***Pa (mm)***	***PET (mm)***	***De Martonne AI***	***Rad ($W\ m^{-2}$)***	***Thermal oscillation (ºC)***
CA (1)	Cambo	France	43º 58'	3º 48' E	610	10.9	20.6	2.4	658	653	-31.5	508	18.2
LE (2)	León	Spain	42° 27'	5° 58' W	871	10.8	29.7	-0.5	505	654	-24.3	566	30.3
MO (3)	Montblanc	Spain	41º 20'	1º 07' E	882	11.3	24.3	2.8	532	662	-25.0	559	21.5
SA (4)	Salamanca	Spain	41° 11'	5° 30' W	886	10.8	25.2	0.7	403	653	-19.4	580	24.5
AT (5)	Atzeneta	Spain	40º 15'	0º 14' W	970	11.6	23.3	2.2	499	669	-23.1	560	21.2
FR (6)	Font Roja	Spain	38º 39'	0º 32' W	1270	14.0	30.2	2.6	420	748	-17.5	575	27.6
SE (7)	Sevilla	Spain	37° 59'	5° 57' W	347	17.1	33.5	5.7	574	890	-21.2	622	27.8
AL (8)	Alhamilla	Spain	36º 59'	2º 23' W	1370	14.5	30.0	5.1	239	767	-9.8	625	24.9
FE (9)	Felix	Spain	36º 54'	2º 40' W	1138	13.3	30.5	0.5	310	730	-13.3	610	30.0
CH (10)	Chaouen	Morocco	35° 05'	5° 05' W	939	13.4	25.1	4.7	647	701	-27.6	604	20.5
BM (11)	Beni Mellal	Morocco	32° 23'	6° 12' W	850	17.2	27.1	8.8	443	849	-16.3	681	18.3
AG (12)	Agadir	Morocco	30° 45'	8° 44' W	1553	21.3	33.5	10.6	309	1136	-9.9	695	22.9

3.2. Field sampling, culture, and growth conditions

The analyses in the 12 populations were conducted in two different non-correlative years owing to the amount of work that these experiments required. We analyzed 6 populations per year following the same experimental protocol. Acorns were collected

during the autumns of 2010 and 2013 (for eastern locations and western locations, respectively). Each year, a mean of 1000 acorns per population were collected from 20 different trees randomly assigned but separated at least 30 m to reduce the likelihood of sampling related trees.

Healthy acorns were labeled and weighed to determine their fresh mass. Labeled acorns were placed in trays with perlite substrate for germination under glasshouse conditions at Pablo de Olavide University, Seville, Spain (37°21'3N, 5°56'7W, 15 m.a.s.l.). Each year, one month after germination, around 100 seedlings per population were randomly taken and sown in 1-liter PVC pots filled with a substrate (3:1 sand:perlite mixture). Seedlings were kept growing in a glasshouse (at a day/night mean temperature of around 19/11ºC, a mean RH of 62%, and a mean photon-flux density of 300 µmol $m^{-2}s^{-1}$) for two months.

When seedlings were roughly 10 cm tall, a mean of 18 plants were randomly taken per population. The seedlings from all populations were pooled and placed in three 1 m^3 growth chambers under the following conditions: day/night temperature of 25/20ºC, day/night RH of 56/80%, photosynthetic photon flux density (PPDF) of 400 µmol $m^{-2}s^{-1}$ at plant height (similar lighting conditions to those experienced by these seedlings in understory), and 11-h photoperiod. Plants were irrigated twice daily with tap water from a drip system to ensure adequate plant water availability. *Hoagland* nutrient solution was administered through the irrigation system twice weekly. To minimize the chamber effect, plants were rotated both inside chamber and between chambers (once weekly).

At the end of the experiment (after three months of growth under optimal and homogeneous conditions), seedlings were separated into its stem, leaf, and root

fractions, and were oven dried at 70ºC until constant weight. Previously, three fresh leaves per seedling were collected to measure mean surface leaf (LS) using image-analysis software *ImageJ*. The specific leaf area (SLA = area/dry weight) and shoot-to-root weight (S/R) were calculated for each seedling. Total leaf area per seedling (LA) was calculated by weighing the total number of leaves and multiplying by individual leaf area. LAR (total leaf-area to plant weight) for each seedling was calculated by dividing total leaf surface by total seedling weight; while LWR (total leaf weight to plant weight) was estimated by dividing the total leaf weight by the total seedling weight.

3.3. Gas exchange and chlorophyll a fluorescence measurements

Gas exchange measurements were made in attached leaves (one leaf per seedling, approximately 15 leaves per population). We repeated the measurement considering the same intact individual leaves at three different times of the experiment (initial time, 50 and 90 days) using an open infra-red gas exchange system (Model CIRAS-I, PP-Systems, Edinburgh, UK). Net photosynthesis (*PN*) and stomatal conductance (*GS*) determinations were performed at ambient CO_2 concentrations (390-400 ppm), 60% of relative humidity, and 450-500 µmol $m^{-2}s^{-1}$ PPFD. Intercellular CO_2 concentration (Ci) was determined following the equations described on Von Caemmerer and Farquhar (1981). Intrinsic water use efficiency (WUEi) was calculated as the ratio between *PN* and *GS*.

After 90 days in growth chambers, one fully developed leaf from the seedling tip was analyzed by modulated chlorophyll *a* fluorescence (n = approximately 15 leaves per population). Measurements were made with a pulse-modulated portable fluorometer

(FMS 2, Hansatech Instruments Ltd, Norfolk UK). For light measurements the selected leaves were adapted to a PPFD of 450 µmol $m^{-2}s^{-1}$ during one hour to ensure that all samples were measured under the same conditions. Subsequently, fluorescence emission (F_s) was carefully recorded on selected leaves during actinic illumination, followed by a saturating pulse that provides the maximum fluorescence during actinic illumination (F_m'). Minimal fluorescence from light-adapted leaf (F_o') was calculated as Oxborough & Baker (1997). Later, the sampled leaf was dark adapted for 30 min in order to the primary quinone acceptor of Photosystem II (Q_A) becomes maximally oxidized, thereby giving the minimal fluorescence from dark-adapted leaf (F_o) upon excitation with a weak non-actinic beam. The maximum fluorescence (F_m) was measured following a pulse of saturating light to maximally reduce Q_A. Variable fluorescence (F_v) was determined as the difference between F_m and F_o, and the maximum quantum yield of Q_A reduction was estimated as the ratio F_v/F_m. The actual quantum yield of Photosystem II (PSII) photochemistry (Φ_{PSII}) was estimated from $(F_m' - F_s)/F_m'$ (Genty *et al.*1989). *NPQ* (non-photochemical quenching) is the fraction of the absorbed light energy dissipated as heat (Verhoeven *et al.* 1996). *NPQ* was determined according to the equation $NPQ = F_m/F_m' - 1$ (Bilger & Björkman 1990). The coefficient of photochemical quenching (q_P) is related to the fraction of open PSII reaction centers, and was estimated as $(F_m'-F_s)/(F_m'-F_o')$ (Schreiber *et al.* 1986). Finally, photochemical quenching (F_q'), related to the amount of electrons flowing through the electron transport chain, was calculated as $F_m'-F_s$ (Baker 2008).

3.4. Statistical analyses

Since experiments were performed in two different years and growth-chamber conditions could not be exactly the same, we normalized all variables considered (see

Methods S2 in Appendix S3 and the original values in Tables S4 and S5 in Appendix S2). Thereby, we avoided finding differences between populations caused by different growth conditions between years.

Gas exchange parameters were analyzed using a repeated measurement ANOVA including *Population* and measurement date (*Time*) as factors. Fluorescence and morphological variables were assessed by a one-way ANOVA including *Population* as factor. Data were transformed when it was necessary to meet the assumptions of the ANOVA. When differences were relevant, an *a posteriori* test was conducted (Tukey's *post hoc* HSD) to make individual comparisons at the population level. The results were analyzed with statistic software STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

Regression analyses were used to investigate relationships between mean physiological and morphological traits of each population and either climatic variables at population origin or latitude (geographic location). A Principal component analysis (PCA) was also performed to cluster the *Q. ilex* populations based on the multivariate relationships among physiological and morphological traits.

4. RESULTS

4.1. Population-level differentiation

Under controlled growth conditions, the analysis of variance revealed highly significant phenotypic differences among *Q. ilex* populations in all traits (gas exchange, chlorophyll *a* fluorescence and morphological variables), except for F_v/F_m (Table 2). No

significant differences among sampling dates (initial time, 50 and 90 days of experiment) were found in the gas exchange measurements; however, the *Populations* x *Time* interaction were strongly significant for all gas exchange variables (Table 2).

Table 2. Repeated measurements ANOVA results for Gas Exchange variables (*PN, GS, Ci* and *WUE*) including Population, Time and their interactions as factors. One-way ANOVA results for Fluorescence (F_v/F_m, Φ_{PSII}, F_q', q_P, *NPQ*) and Morphological variables (Total mass, SLA, LS, LA, S/R, LAR) also are given. Letters in bold denote significant differences ($P < 0.05$).

	POPULATION		**TIME**		**POP X TIME**	
	F	*P*	*F*	*P*	*F*	*P*
PN	6.97	**<0.001**	0.32	0.723	1.90	**<0.01**
GS	8.66	**<0.001**	0.20	0.821	4.07	**<0.001**
Ci	5.96	**<0.001**	0.07	0.934	5.80	**<0.001**
WUE	7.10	**<0.001**	0.06	0.942	4.01	**<0.001**
$\boldsymbol{F_v/F_m}$	0.47	0.919		-		-
$\boldsymbol{\Phi_{PSII}}$	3.02	**<0.001**		-		-
$\boldsymbol{F_q'}$	4.17	**<0.001**		-		-
$\boldsymbol{q_P}$	3.02	**<0.001**		-		-
NPQ	3.36	**<0.001**		-		-
Total mass	6.06	**<0.001**		-		-
SLA	14.17	**<0.001**		-		-
LS	8.13	**<0.001**		-		-
LA	6.64	**<0.001**		-		-
S/R	4.93	**<0.001**		-		-
LAR	8.83	**<0.001**		-		-

Considering the mean normalized values of the three sampling dates of gas exchange, seedlings from the Agadir and Cambo populations (the southernmost and the northernmost populations of distribution range, respectively) showed significantly the lowest *PN* values, followed by Chaouen; while Alhamilla, Salamanca and León showed the highest values for this variable ($P < 0.001$, Fig. 1a). Stomatal conductance (*GS*) also showed significant differences among populations with the lowest values in Agadir, Cambo, and Chaouen seedlings, while León, Sevilla, and Felix had the highest values ($P < 0.001$, Fig. 1b).

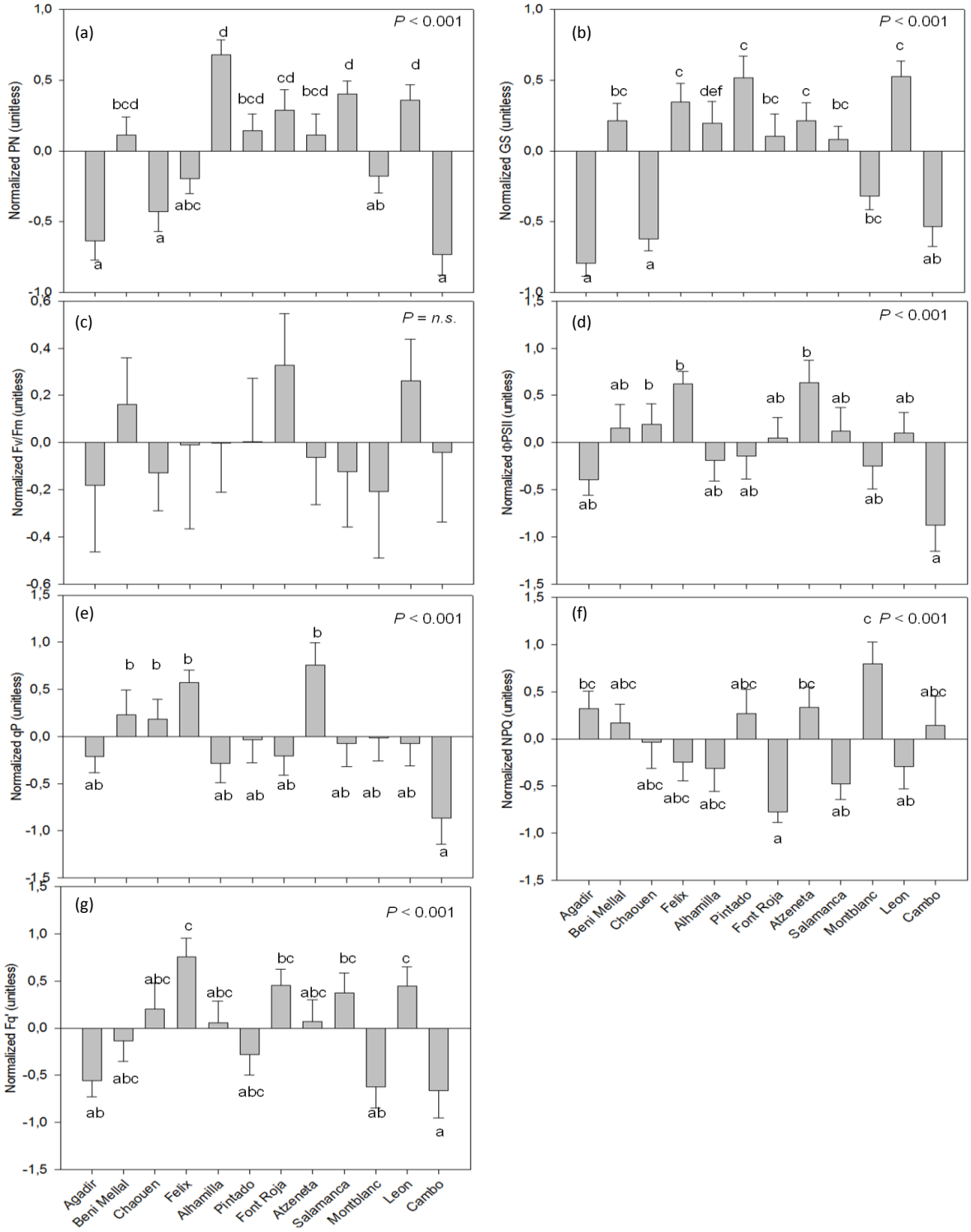
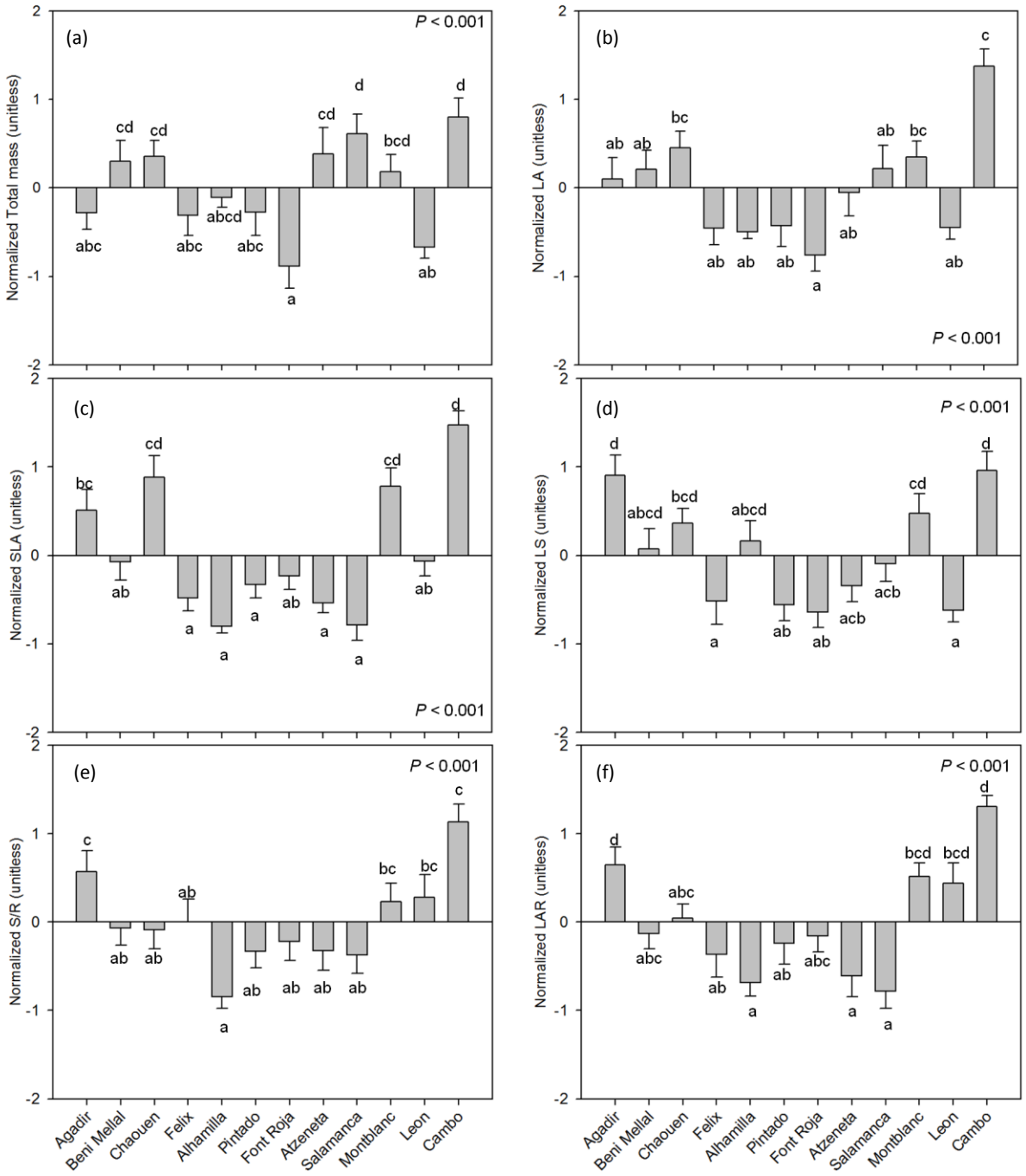


Figure 1. Population differences in mean normalized values (± SE) of physiological traits related to photosynthetic performance in seedlings growing inside growth chambers: (a) Photosynthetic rate (*PN*), (b) Stomatal conductance (*GS*) –*PN* and *GS* averaged of all time measurements–, (c) Maximum photochemical efficiency of PSII (F_v/F_m), (d) actual photochemical efficiency of PSII (Φ_{PSII}), (e) fraction of opened PSII reaction centers (q_P), (f) non-photochemical quenching (*NPQ*), (g) photochemical quenching (F_q') –fluorescence variables measured at 90 days–. Populations are sorted from south (left) to north (right) of *Q. ilex* distribution range. Different letters denote significant different groups following Tukey *post hoc* analysis.

Note: Bar plots do not represent real distances between populations. Positive bars indicate values above the year mean value for each trait, while negative bars indicate values below the year mean value.



leaf area per seedling (LA), (c) specific leaf area (SLA), (d) leaf size (LS), (e) shoot to root ratio (S/R), (f) leaf area ratio (LAR). Populations are sorted from south (left) to north (right) of *Q. ilex* distribution range. Different letters denote significant different groups following Tukey *post hoc* analysis.

Note: Bar plots do not represent real distances between populations. Positive bars indicate values above the year mean value for each trait, while negative bars indicate values below the year mean value.

On average, the Cambo population showed significantly the lowest normalized values in the main variables relative to the actual efficiency of PSII (Φ_{PSII}, q_P, and F_q'), followed by the Agadir population (except q_P) and, to a lesser extent, the Montblanc population. By contrast, the Atzeneta and Felix populations exhibited the highest normalized values in such variables (except F_q' values in the Atzeneta population) (Fig. 1d,e,g). On the other hand, the Montblanc population showed significantly the highest normalized values for energy dissipated as heat (*NPQ*), while the Font Roja population had significantly the lowest ones (Fig. 1f).

Plants from the northernmost population (Cambo) had the biggest seedlings, with biggest leaf size (LS, LA) and specific leaf area (SLA); whereas Font Roja had the smallest seedlings (Fig. 2a-d). Besides, Cambo together with the southernmost population (Agadir), showed significantly higher allocation to aerial fraction related to roots (S/R) and leaf area ratio (LAR) than core populations (Fig. 2e,f).

The PCA revealed a group of populations characterized by higher photosynthetic efficiency and lower seedling size in the right part of the Axis 1 (especially core populations), compared with edge populations (plus Montblanc and Chaouen), located in the left part of the Axis 1 (see Fig. S2 in Appendix S1).

4.2. Physiological, morphological and climatic relationships

Fig. 3 displays the most significant correlations found between the functional traits and the climatic characteristics at population origin (see Table S6 in Appendix S2 for the complete correlation analyses results). A significant negative linear correlation was detected between the normalized values of Photochemical quenching (F_q') and minimum temperature in spring (Fig. 3a; R^2 = 0.36, P < 0.05), while a positive

correlation was noticed between F_q' and the range of thermal oscillation at the location of origin for each population (Fig. 3b; R^2 = 0.41, P < 0.05). On the other hand, total seedling mass (R^2 = 0.62, P < 0.01) and total leaf area –LA– (R^2 = 0.65, P < 0.01) were negatively correlated with the range of thermal oscillation in the habitat of each population (Fig. 3c,d). In addition, the mean normalized values of SLA showed a positive relationship with annual precipitation at site of origin (Fig. 3e; R^2 = 0.41, P < 0.05).

4.3. Geographic patterns of variation

The normalized data of *PN* were related marginally to geography (Fig. 4a): In all gas exchange determinations (initial time, 50 and 90 days) edge populations' *PN* was lower than those observed in populations native to the core of the distribution range. Similarly, an almost significant hyperbolic relationship was found between latitude of origin and the mean values of normalized data of light-fluorescence variables *ΦPSII* and q_P (Fig. 4b,c). In addition, leaf size (LS) and structural traits (S/R and LAR) showed a significant hyperbolic pattern, with the highest values in populations located in the boundaries of the species distribution range (Fig. 4d,e,f).

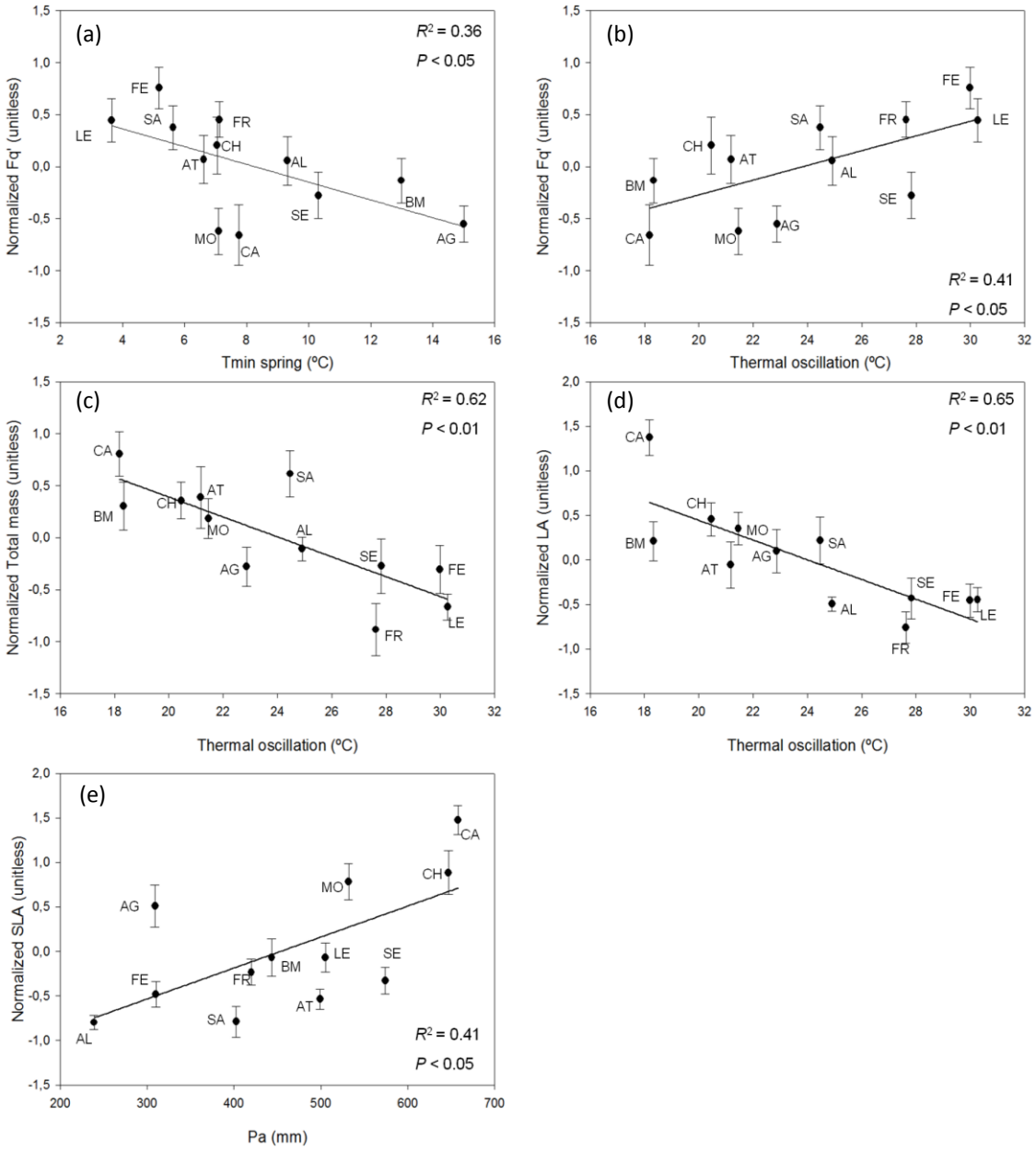


Figure 3. Correlations between climatic variables at each population site with physiological or morphological variables. Population mean normalized values ± SE (n = 15-20 per population), R^2 and P values are shown. Study populations codes in Table 1.

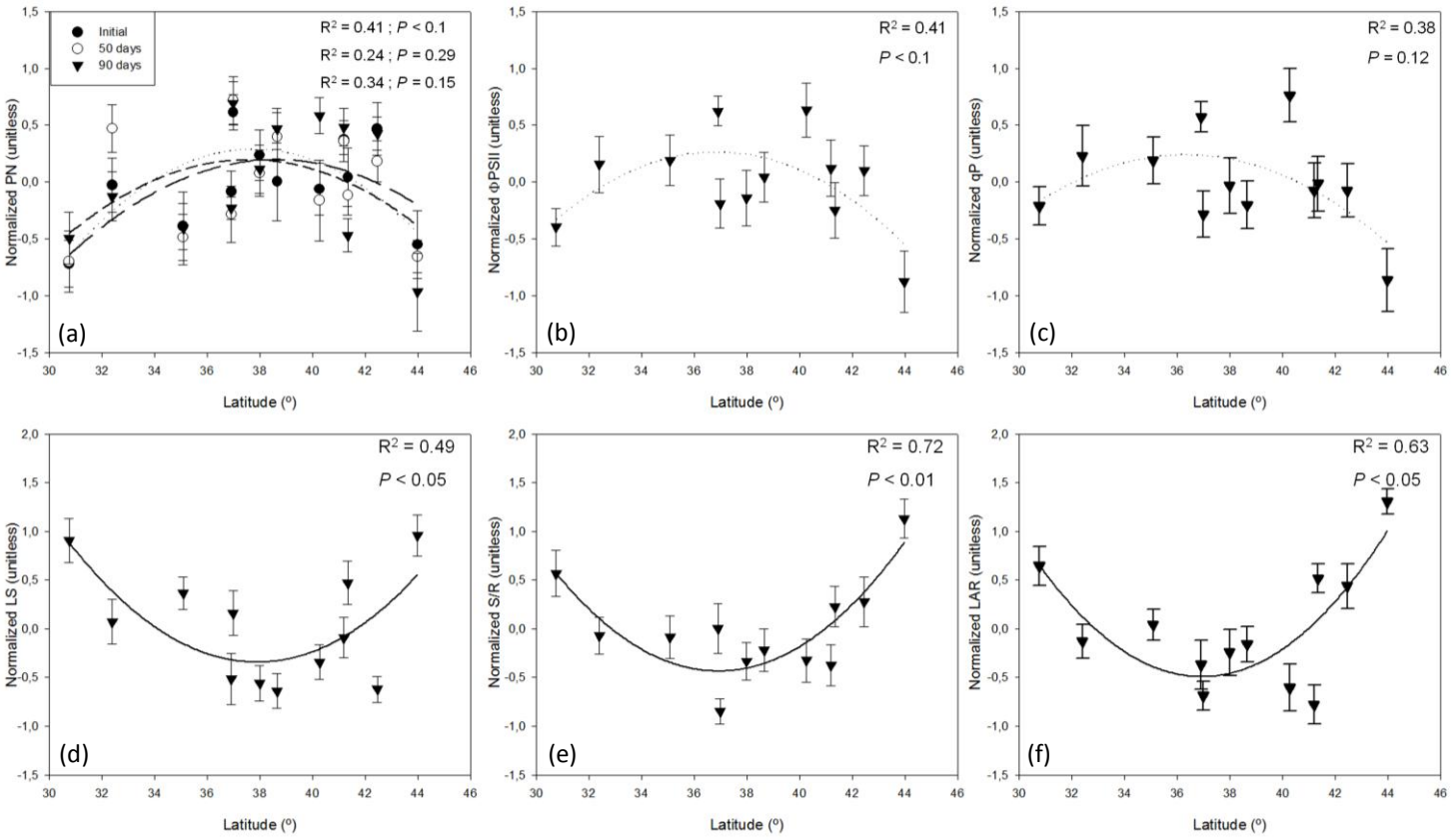


Figure 4. Relationships between latitude of origin and mean normalized data of (a) photosynthetic rate (*PN*) at different growing times, (b) actual PSII efficiency (Φ_{PSII}), (c) fraction of opened PSII reaction centers (q_P), (d) leaf size (LS), (e) shoot to root ratio (S/R) and (f) total leaf area to plant mass (LAR) for each population at 90 days of experiment (± SE). Symbols represent different date samples: black circles, initial; white circles, 50 days; and black triangles, 90-days sampling.

5. DISCUSSION

Quercus species are known to exhibit high inter-population variability in morphological, structural, and physiological traits throughout environmental gradients (Gratani *et al.* 2003; Bruschi 2010; Ramírez-Valiente *et al.* 2010; García-Nogales *et al.* 2016), although the relationships among these components of species´ adaptive capacity remained poorly explored. As regards *Q. ilex* case study, such inter-population variability likely arises from complex interactions between the species' genetic diversity (Lumaret *et al.* 2002) and the selective pressure imposed by the wide range of environmental conditions, particularly climatic and edaphic, where this species grows (Barbero *et al.* 1992; Laureano *et al*. 2008 and 2013).

We found substantial phenotypic differentiation among *Q. ilex* populations. Specifically, among-population variability for those traits related to chlorophyll *a* fluorescence, carbon assimilation and seedling morphology was highly significant. Some photosynthetic and morphological traits were significantly related to climate (Fig. 3), or spatially structured according to the location of origin (core *vs.* edge population differences; Fig. 4). These patterns suggest that population differentiation might be related to constitutive morphological and physiological traits according to local adaptation.

5.1. Population divergence and correspondence to local origin

We found weak relationships among physiological traits and climatic variables over the different *Q. ilex* populations studied here (Table S6 in Appendix S2). Only the Photochemical quenching (F_q') showed a negative correlation with the minimum temperature of spring and a positive correlation with the range of thermal oscillation in

the habitat of origin (Fig. 3a,b). This finding indicates that populations native to cooler habitats and characterized by a shorter growing season allocate higher amount of electrons to the electronic transport chain (i.e. they display higher F_q' values). Higher photosynthetic rate (which is related to F_q', see Fig. S4 in Appendix S1) could offset the shorter growing season of populations native to the coldest regions (Oleksyn *et al.* 1998; Benowicz *et al.* 2000). Furthermore, higher foliar nitrogen content has been reported at populations native to low temperatures sites, including oaks, suggesting a higher investment in photosynthetic endowments (Reich & Oleksyn 2004; Bresson *et al.* 2011; Atkin *et al.* 2015). Besides, *Q. ilex* populations native to warm habitats showed under homogeneous growth conditions lower amount of electrons transmitting through the electron transport chain (Table 1 and Fig. 3a), which may indicate a drought adaptation (Balaguer *et al.* 2002) Thus, low water availability is related to stomatal closure and a lower carbon gain, which would stimulate free-radical production in the case of electron-flow excess (Aranda *et al.* 2005). In summary, the relationship obtained here between F_q' and habitat minimum temperature suggests a constitutive local adaptation of F_q' to the habitat of origin, which prevails over the plasticity under common growth conditions. Although there are some limitations using F_q' values, as chlorophyll fluorescence absolute values may be very variable, we consider this parameter useful to asses among-groups variability because we are using similar leaves for size, greenness and health conditions.

As regards the above-mentioned relationship between the electron flow and temperature, the northwestern population (León), which is characterized by both the highest range of thermal oscillation and the lowest minimum winter temperature, supports the hypothesis of higher photochemical quenching (F_q') at populations native

to low temperatures sites. On the other hand, the warmest population (Agadir) shows quite low *PN* values (as well as related variables). On the contrary, it is noticeable that the northernmost population (Cambo) yielded values lower than expected, both for the photosynthetic rate as well as for photochemical efficiency (Φ_{PSII} and F_q'). Here, limited photosynthetic performance in Cambo might be explained partially by the occurrence of less cold conditions, compared to other locations, this likely being a buffering effect of its proximity to the Mediterranean Sea.

The significant positive relationship found between seedling total mass or total leaf area and the range of thermal oscillation at the habitat of origin (Fig. 3c,d) seems to mismatch with the correlation previously described for F_q'. In other words, the populations characterized by broader thermal fluctuation along the year (and perhaps a shorter growing season) were more efficient in terms of photochemical performance, but they did not yield the biggest seedlings. Some authors also have reported negative correlations between growth variables and photosynthetic performance, suggesting that differences in the length of the growing period had a larger impact on biomass accumulation than differences in net photosynthesis (Benowicz *et al.* 2000). This finding could be explained owing to locations with a wide range of thermal oscillation might allocate higher energy to the tolerance of extreme temperatures, which constrains the allocation to growth, resulting in a conservative resource-use strategy (Koehler *et al.* 2012; Savage & Cavender-Bares 2013).

The relationship found between SLA and annual precipitation agrees with previous studies for several species reporting that SLA decreases in water-limited populations (Bussotti *et al.* 2002; Marchin *et al.*, 2008; Ramírez-Valiente *et al.* 2010), as leaves characterized by thick cell walls (i.e. low SLA) contribute to an efficient

control of gas exchange, avoiding the water loss by transpiration (Bussotti *et al.* 2002). Besides, low SLA is considered as an important strategy for plants living in resource-poor and harsh environments (Reich *et al.* 1999 and references therein), which is usually associated with low photosynthetic capacity. In our study, plants native to edge populations (i.e. the putative harshest environments within the species range) showed high values of SLA, while *PN* values were the lowest (Fig. S5d in Appendix S1). As a consequence, our results do not support a positive relationship between SLA and photosynthetic rate, as other studies report (Reich *et al.* 1999, Wright *et al*. 2004; Shipley *et al*. 2005).

Regarding other physiological traits, it would be expected that populations subjected to contrasting environmental conditions, such as water availability, temperature or radiation intensity, show differences in light-energy harvesting capacity, assuming that this process is subjected to a selective pressure (Aranda *et al.* 2005; Corcuera *et al.* 2011). Nonetheless, contrary to expectations, maximum PSII photochemical efficiency (F_v/F_m), which is related to efficiency in absorbing light, showed no significant differences among populations (Table 2). This lack of differentiation in F_v/F_m disagrees with other studies on *Quercus* species, which reported significant intra-specific F_v/F_m variability (Aranda *et al.* 2005; Cavender-Bares 2007). Generally, F_v/F_m seems to show a quite constant ratio (about 0.8) when populations grows in a stress-free environment (Baker 2008). Indeed, when the plants studied here were exposed to stressful conditions (e.g. water shortage), they showed significant among-population F_v/F_m differences (García-Nogales, unpublished). All together, this suggests that *Q. ilex* maximum PSII photochemical efficiency could be considered a

plastic trait that depends on the environmental conditions to which individuals are exposed (Valladares *et al*. 2002).

By the opposite, we observed significant inter-population differences in *NPQ* (Table 2 and Fig. 1f), suggesting that not all populations release the excess of light energy as heat in the same proportion; however, we found no relation between *NPQ* and the climate at the site of origin. On the other hand, we found that populations differed in the water-use efficiency, exhibiting a slight latitudinal trend, where the southernmost population (Agadir) registered the highest value together with the lowest intercellular CO_2 (Fig. S3 in Appendix S1). This result was expected because the evolution in habitats characterized by strong water shortage, as in such population (high aridity index and the highest potential evapotranspiration; Table 1), would result in a disproportionately low stomatal conductance (Goisser *et al*. 2013; Lázaro-Nogal *et al.* 2015). The persistence of this trait under non-limiting conditions suggests a constitutive adaptation according to origin climatic conditions. This agrees with ongoing research of tree-rings, which displays the lowest $\delta^{13}C$ discrimination in trees native to the southernmost populations growing under natural conditions (unpublished data).

5.2. Range margin differences among populations

After three months of experiment, populations reflected some range margin trends where seedlings native to the periphery of the distribution range exhibited comparatively low values of *PN*, q_P and Φ_{PSII}, whereas seedlings native to presumably less stressful environments (core populations) exhibited higher photosynthetic efficiency under common growth conditions (Fig. 4a-c). For *PN*, this range margin trend was registered in all the determinations performed over the experiment (initial time, 50 and 90 days), suggesting a robust pattern (Fig. 4a). On the other hand, the

opposite hyperbolic relationships were observed between the latitude of origin and morphological traits (LS, S/R, LAR) (Fig. 4d-f). Consequently, it might be inferred a negative relationship between the photosynthetic and these morphological traits (see Fig. S5a,b,c in Appendix S1), where the populations with higher investment in aerial fraction exhibited lower photosynthetic efficiency in terms of *PN*. This finding agrees with the negative relationship between LAR and *PN* reported by other study on woody species (Liu *et al.* 2011). However, and contrary to what has been found, low LAR (derived from low LS values) should be expected as environmental stress rises (i.e. at species edge ranges), since small leaves could work better under both water limitations and cold environments (Laureano *et al.* 2016).

Even though the underlying mechanisms of the relationship between the photosynthetic performance and plant structural traits are not well known, we suggest that the low photosynthetic efficiency found within edge populations (Fig. 4a-c) could be adaptive. Thus, plants from stressful environments could invest few resources to photochemical performance owing to: i) more resource-limited habitats (Laureano *et al.* 2013), ii) higher energy allocation for defense against stress and recovery systems (Takashima *et al.* 2004), or iii) higher photochemical efficiency may produce reactive oxygen species (ROS) and further photodamage, for instance in habitats subjected to high irradiance (southern-edge populations) (Demmig-Adams *et al.* 1996) or on cooler and cloudless days in stressful habitats such as the northern edge (Aranda *et al.*, 2005). Although these three statements could take place simultaneously, we conclude that the last two are more plausible regarding the higher respiration rates in edge populations compared with core ones (García-Nogales, unpublished), which suggest that edge-range populations have evolved under more stressful environments (Laureano *et al.* 2016).

Therefore, the negative relationship between photosynthetic and structural traits would be explained as a compensation mechanism, in which populations with low photosynthetic efficiency would invest higher resources to the aerial fraction. This significant hyperbolic pattern in plant structure related to latitude of origin agrees with the previous findings in these populations, in which the seedlings native to edge populations seem to promote the allocation to aerial biomass (higher S/R values) in order to maximize the energy uptake during the limited period favorable to growth (García-Nogales *et al.* 2016).

It bears noting that the two peripheral populations reached the lowest values of both *PN* and Φ_{PSII}, but this was achieved through different ways. Hence, the northernmost population (Cambo) registered the lowest fraction of open PSII reaction centers (q_P), as well as a modest heat dissipation (*NPQ*), signifying a regulation strategy based essentially on fluorescence emission. Meanwhile the southernmost population (Agadir) combined relatively high *NPQ* values together with modest q_P values. In both populations such mechanisms resulted in the lowest actual PSII efficiency (Φ_{PSII}), leading these two populations to transmit a low amount of electrons through the electron transport chain (F_q', Fig. 3e). This in turn resulted in the lowest values of *PN* registered for the 12 populations (Fig. 2a).

Our outcomes support the idea that constitutive adaptive shifts to stressful environments (such as edge populations Cambo and Agadir) may be related to a conservative resources-use strategy in response to the limiting environmental conditions in their natural habitats, even under plentiful resources conditions (Chapin 1980; Baquedano *et al.* 2008); while the core populations had a more efficient photosynthetic apparatus, enabling them to take better advantage of available resources. Besides, the

lower intrinsic efficiency in both light use (Φ_{PSII} and F_q') and carbon fixation (PN) in populations native to species' range edges could be also related to a resistance strategy that would enable the continuity of the biochemical and physiological reactions under extreme environmental conditions, typical of stressful environments (low maximum temperatures throughout the year in the north and low water availability in the south). In addition, the higher maintenance respiration rate characteristic of stressful environments (Laureano *et al.* 2008, 2013 and 2016) would contribute to a lower net photosynthetic rate (Chapin 1993).

5.3. Conclusions

Substantial among-population variability appears in seedlings growing under non-limiting experimental conditions; suggesting that the genotypic variation overrides the phenotypic plasticity in gas exchange and fluorescence (except for F_v/F_m, which reflected a high acclimation capacity). The lack of maternal effect (no relationship between functional traits and acorn mass) suggests that the interpopulation variability might be attributed to local adaptation. For example, the amount of electrons flowing through the electronic transport chain was determined by minimum temperature in the climate of origin.

Given the more favorable environmental conditions at the core of the distribution range, it is likely that there was less selective pressure for differentiation in photosynthetic performance compared to stress-induced selective pressures under range-edges limiting environmental conditions. The low-efficiency light processing by seedlings native to edge populations (which could be related to low phenotypic plasticity) supports the hypothesis stating that peripheral locations do select for individuals following a phenotypic canalization strategy, and therefore, could be better

adapted to withstand more extreme climatic conditions for the species. This may imply that individuals are inherently more tolerant to stress and better able to cope with constraining environmental conditions than are individuals native to other origins. This finding emphasizes the importance of take into consideration population differentiation in key functional traits in order to predict future species' distribution shifts in response to the adverse conditions that climate change will impose on Mediterranean species.

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7. SUPPORTING INFORMATION

Appendix S1. Supplemental figures S1-S4

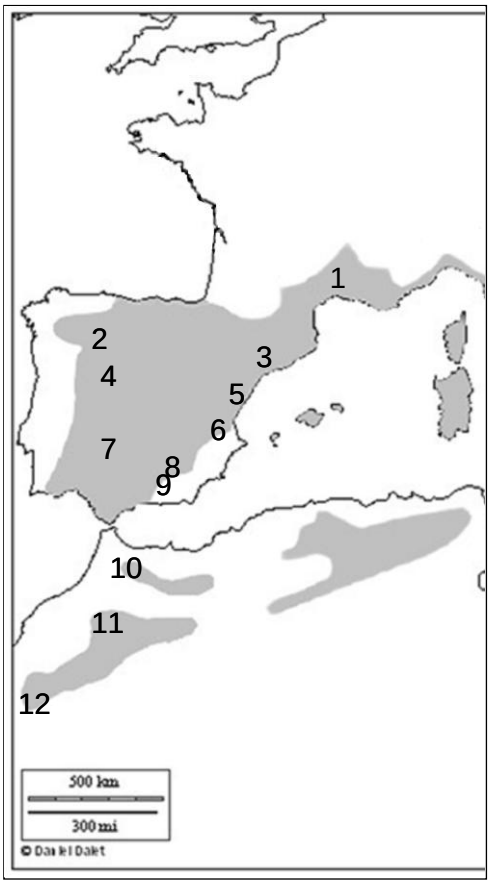

Fig. S1 Locations of the 12 sampled populations in the western part of *Q. ilex* distribution range (map modified from Delzon *et al.*, 2013). Numbers denoting each population are detailed in Table 1.

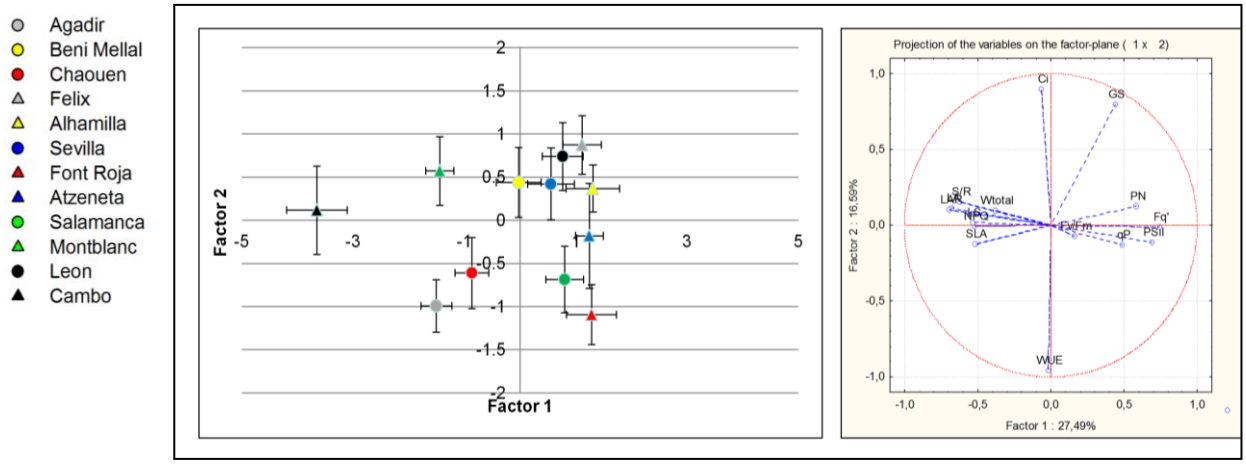

Fig. S2 Results of the PCA showing the mean values (± SE) of the first and second factor coordinates for each population. Also is displayed the projection of the variables for each factor and the percentage of variance accounted by each factor is noted.

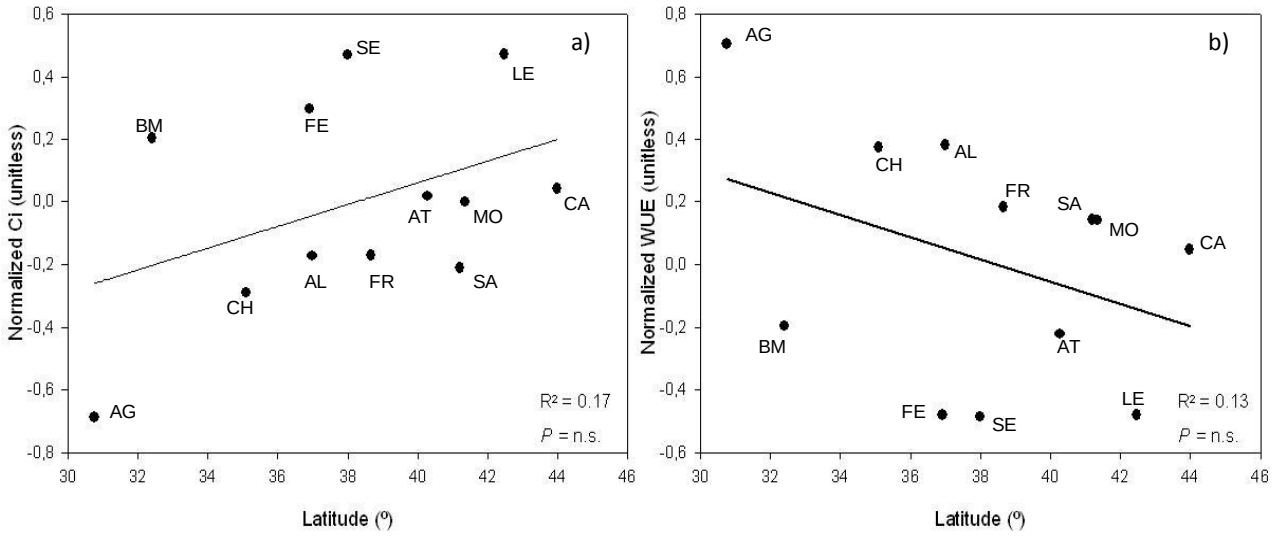

Fig. S3 Relationship between latitude of origin and mean normalized data for intercellular CO_2 concentration (Ci) (a) and water use efficiency (WUE) (b) analyzed in seedlings of the 12 *Q. ilex* populations raised in growth chambers (all measurement times combined). Study populations codes in Table 1.

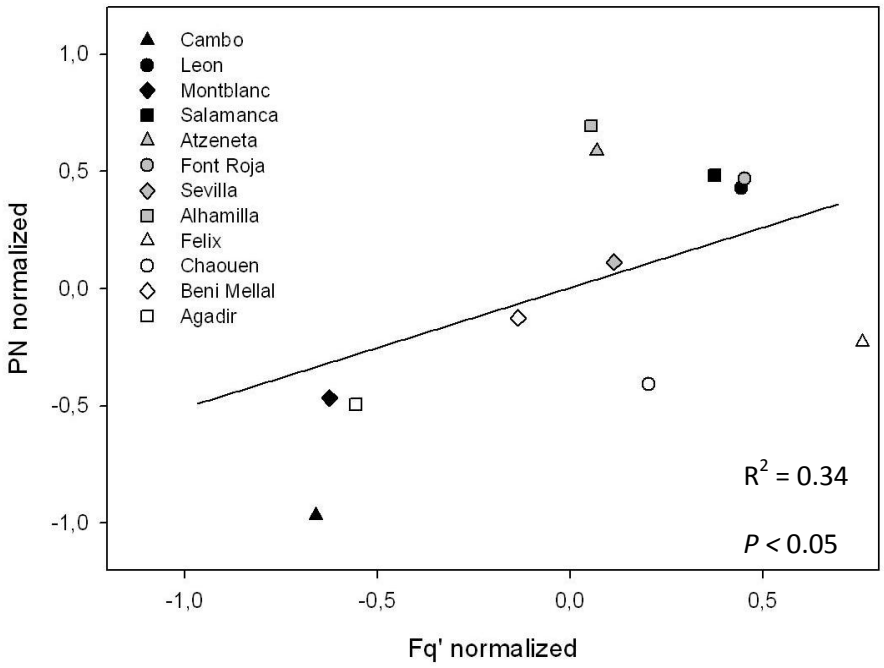

Fig. S4 Relationship between mean *PN* normalized values against mean F_q' normalized values in seedlings of the 12 *Q. ilex* populations at 90 days in growth chambers. Populations are listed from North (top) to South (bottom).

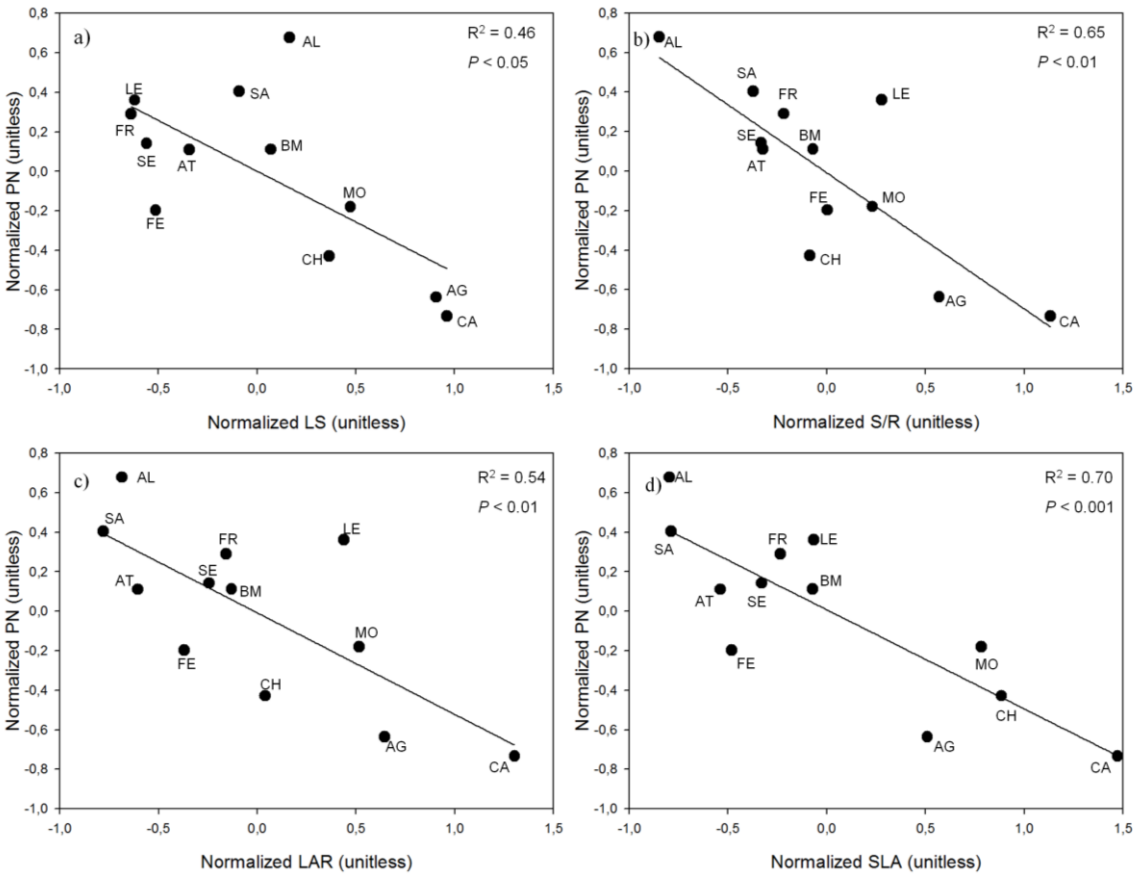

Fig. S5 Relationship between mean normalized values of leaf size (LS) (a), shoot to root ratio (S/R) (b), total leaf area ratio (LAR) (c), and specific leaf area (SLA) (d) against mean *PN* normalized values (all measurement times combined) in seedlings of the 12 *Q. ilex* populations.

Appendix S2. Supplemental tables S1-S7

Table S1 Mean annual and seasonal temperature of the 12 populations of *Q. ilex* considered.

Location	Latitude	Tmean	Tmean fall	Tmean win	Tmean spring	Tmean sum
Cambo	43.98	10.9	11.5	3.9	9.8	18.3
León	42.46	10.8	11.6	2.9	9.4	19.4
Montblanc	41.34	11.3	12.2	4.2	9.8	19.1
Salamanca	41.19	10.8	11.4	2.7	9.3	19.8
Atzeneta	40.27	11.6	12.5	4.5	9.7	19.6
Font Roja	38.66	14.0	15.1	6.5	11.7	22.5
Sevilla	37.99	17.1	18.0	9.2	15.4	25.7
Alhamilla	36.99	14.5	16.0	6.7	12.1	23.2
Felix	36.91	13.3	14.6	5.1	10.8	22.7
Chaouen	35.09	13.4	14.6	8.4	11.3	19.3
Beni Mellal	32.39	17.2	18.0	11.1	15.5	24.1
Agadir	30.76	21.3	21.9	15.1	19.7	28.6

Table S2 Mean maximum annual and seasonal temperature of the 12 populations of *Q. ilex* considered.

Location	Latitude	Tmax mean	Tmax fall	Tmax win	Tmax spring	Tmax sum
Cambo	43.98	12.9	13.2	5.6	12.0	20.6
León	42.46	18.3	18.8	7.6	16.9	29.7
Montblanc	41.34	14.7	15.4	5.9	13.3	24.3
Salamanca	41.19	14.6	15.1	5.0	13.2	25.2
Atzeneta	40.27	14.6	15.3	7.0	13.0	23.3
Font Roja	38.66	20.3	21.3	11.8	18.1	30.2
Sevilla	37.99	23.2	23.8	14.0	21.5	33.5
Alhamilla	36.99	18.7	20.3	8.7	15.8	30.0
Felix	36.91	20.0	21.1	11.1	17.5	30.5
Chaouen	35.09	18.1	19.0	12.2	16.0	25.1
Beni Mellal	32.39	19.7	20.2	13.2	18.2	27.1
Agadir	30.76	26.5	26.7	20.6	25.0	33.5

Table S3 Mean minimum annual and seasonal temperature of the 12 populations of *Q. ilex* considered.

Location	Latitude	Tmin mean	Tmin fall	Tmin win	Tmin spring	Tmin sum
Cambo	43.98	9.1	10.1	2.4	7.8	16.2
León	42.46	5.1	6.0	-0.5	3.7	11.1
Montblanc	41.34	8.8	9.8	2.8	7.1	15.4
Salamanca	41.19	7.3	8.2	0.7	5.6	14.8
Atzeneta	40.27	8.7	10.0	2.2	6.6	16.2
Font Roja	38.66	9.5	10.9	2.6	7.1	17.5
Sevilla	37.99	12.0	13.3	5.7	10.3	18.7
Alhamilla	36.99	11.4	12.9	5.1	9.3	18.4
Felix	36.91	7.5	8.9	0.5	5.2	15.4
Chaouen	35.09	9.1	10.4	4.7	7.0	14.4
Beni Mellal	32.39	14.7	15.7	8.8	13.0	21.1
Agadir	30.76	16.7	17.6	10.6	15.0	23.4

Table S4 Mean values (± SE) of gas exchange and fluorescence variables in the 12 *Q. ilex* populations after 90 days of experiment.

Population	***PN***	***GS***	***Ci***	***WUE***	F_v/F_m	Φ_{PSII}	F_q'	q_P	***NPQ***	***n***
Cambo	13.2 ± 0.7	176.0 ± 9.3	229 ± 7	0.077 ± 0.003	0.814 ± 0.004	0.268 ± 0.015	152 ± 12	0.363 ± 0.020	0.579 ± 0.092	15
León	13.6 ± 0.4	110.2 ± 7.8	191 ± 13	0.135 ± 0.010	0.818 ± 0.002	0.329 ± 0.012	186 ± 11	0.462 ± 0.019	0.817 ± 0.130	19
Montblanc	14.3 ± 0.3	205.9 ± 6.7	252 ± 3	0.070 ± 0.002	0.811 ± 0.004	0.303 ± 0.014	154 ± 9	0.426 ± 0.018	0.769 ± 0.069	15
Salamanca	13.9 ± 0.5	81.3 ± 5.4	139 ± 12	0.183 ± 0.008	0.813 ± 0.003	0.331 ± 0.014	183 ± 11	0.462 ± 0.019	0.717 ± 0.089	20
Atzeneta	16.5 ± 0.3	224.1 ± 6.7	232 ± 3	0.073 ± 0.002	0.813 ± 0.003	0.353 ± 0.013	183 ± 10	0.484 ± 0.017	0.635 ± 0.065	16
Font Roja	16.3 ± 0.4	199.8 ± 8.8	214 ± 5	0.083 ± 0.004	0.819 ± 0.003	0.319 ± 0.012	199 ± 7	0.412 ± 0.016	0.312 ± 0.033	15
Sevilla	12.7 ± 0.7	102.3 ± 10.9	187 ± 17	0.144 ± 0.012	0.815 ± 0.004	0.316 ± 0.014	149 ± 12	0.466 ± 0.020	1.118 ± 0.141	20
Alhamilla	16.7 ± 0.4	232.9 ± 11.6	243 ± 4	0.074 ± 0.003	0.814 ± 0.003	0.306 ± 0.012	182 ± 10	0.406 ± 0.015	0.447 ± 0.071	14
Felix	14.8 ± 0.2	219.7 ± 9.9	240 ± 6	0.069 ± 0.003	0.814 ± 0.005	0.352 ± 0.007	212 ± 8	0.470 ± 0.010	0.467 ± 0.058	14
Chaouen	11.1 ± 1.0	62.5 ± 6.1	152 ± 22	0.183 ± 0.012	0.813 ± 0.002	0.335 ± 0.012	174 ± 14	0.483 ± 0.017	0.953 ± 0.148	17
Beni Mellal	12.0 ± 0.7	95.9 ± 9.2	191 ± 15	0.141 ± 0.012	0.817 ± 0.003	0.333 ± 0.014	156 ± 11	0.487 ± 0.021	1.067 ± 0.107	20
Agadir	10.9 ± 0.7	59.0 ± 4.0	128 ± 15	0.189 ± 0.010	0.812 ± 0.004	0.302 ± 0.009	134 ± 9	0.451 ± 0.013	1.147 ± 0.102	20

Table S5 Mean values (± SE) of morphological traits in seedlings of the 12 *Q. ilex* populations after 90 days of experiment.

Population	***Acorn fresh mass (g)***	***LS (cm^2)***	***LW (g)***	***SLA (cm^2g^{-1})***	***Leaves (g)***	***Shoot (g)***	***Roots (g)***	***Wtotal (g)***	***LA (cm^2)***	***LAR (cm^2g^{-1})***	***LWR (gg^{-1})***	***S/R (gg^{-1})***	***n***
Cambo	1.99 ± 0.13	7.69 ± 0.36	0.087 ± 0.005	89.8 ± 1.7	4.44 ± 0.26	7.30 ± 0.42	2.64 ± 0.17	9.93 ± 0.56	392 ± 22	40.0 ± 1.0	0.448 ± 0.013	2.83 ± 0.15	15
León	2.68 ± 0.15	4.92 ± 0.26	0.075 ± 0.004	65.8 ± 1.1	1.69 ± 0.13	2.51 ± 0.18	2.04 ± 0.23	4.55 ± 0.34	111 ± 9	25.0 ± 1.3	0.382 ± 0.021	1.39 ± 0.12	20
Montblanc	2.70 ± 0.18	6.85 ± 0.40	0.084 ± 0.006	82.5 ± 2.3	3.42 ± 0.23	5.58 ± 0.38	2.72 ± 0.20	8.30 ± 0.52	280 ± 21	34.1 ± 1.2	0.415 ± 0.015	2.15 ± 0.16	15
Salamanca	4.34 ± 0.34	5.97 ± 0.41	0.097 ± 0.007	61.1 ± 1.1	2.48 ± 0.28	4.20 ± 0.44	3.91 ± 0.25	8.11 ± 0.61	156 ± 18	17.9 ± 1.1	0.294 ± 0.018	1.08 ± 0.10	20
Atzeneta	3.72 ± 0.38	5.45 ± 0.31	0.080 ± 0.005	68.4 ± 1.2	3.46 ± 0.41	5.57 ± 0.65	3.27 ± 0.23	8.84 ± 0.78	235 ± 28	25.6 ± 1.8	0.378 ± 0.027	1.73 ± 0.17	14
Font Roja	1.54 ± 0.11	4.87 ± 0.31	0.069 ± 0.004	70.5 ± 1.9	2.27 ± 0.33	3.58 ± 0.54	1.92 ± 0.17	5.50 ± 0.68	157 ± 20	29.0 ± 1.4	0.407 ± 0.017	1.81 ± 0.17	15
Sevilla	5.36 ± 0.47	5.04 ± 0.36	0.079 ± 0.006	64.1 ± 1.0	1.78 ± 0.25	2.81 ± 0.41	2.83 ± 0.36	5.65 ± 0.73	112 ± 15	21.0 ± 1.4	0.328 ± 0.021	1.10 ± 0.09	20
Alhamilla	4.83 ± 0.42	6.32 ± 0.39	0.096 ± 0.006	65.6 ± 0.9	2.84 ± 0.13	4.21 ± 0.18	3.33 ± 0.23	7.54 ± 0.30	186 ± 9	24.9 ± 1.1	0.380 ± 0.016	1.34 ± 0.10	15
Felix	2.46 ± 0.25	5.15 ± 0.45	0.075 ± 0.007	69.0 ± 1.5	2.92 ± 0.33	4.63 ± 0.50	2.38 ± 0.19	7.02 ± 0.60	191 ± 21	27.3 ± 1.9	0.404 ± 0.024	1.98 ± 0.19	15
Chaouen	3.82 ± 0.19	6.87 ± 0.33	0.093 ± 0.006	72.0 ± 1.6	2.34 ± 0.21	3.99 ± 0.33	3.40 ± 0.23	7.39 ± 0.49	172 ± 13	22.7 ± 0.9	0.316 ± 0.016	1.22 ± 0.10	17
Beni Mellal	5.89 ± 0.48	6.29 ± 0.45	0.097 ± 0.008	65.7 ± 1.4	2.37 ± 0.21	3.90 ± 0.36	3.34 ± 0.31	7.25 ± 0.64	155 ± 15	21.7 ± 1.0	0.331 ± 0.016	1.22 ± 0.09	20
Agadir	3.69 ± 0.29	7.95 ± 0.45	0.116 ± 0.007	69.5 ± 1.5	2.14 ± 0.24	3.35 ± 0.36	2.27 ± 0.21	5.63 ± 0.52	148 ± 16	26.2 ± 1.2	0.377 ± 0.015	1.52 ± 0.11	20

Table S6 Pearson correlation coefficients between physiological or morphological traits and climatic characteristics at population origin.

	PN	***GS***	***Ci***	***WUE***	***Fv/Fm***	***Fq'***	***ΦPSII***	***qP***	***NPQ***	***Total mass***	***SLA***	***LS***	***LA***	***S/R***	***LAR***	***LWR***
Latitude	0.16	0.24	0.42	-0.37	0.09	0.04	-0.15	-0.26	-0.09	0.19	0.08	-0.18	0.20	0.18	0.17	0.12
T	-0.20	-0.20	-0.34	0.32	-0.05	-0.30	-0.15	-0.01	0.18	-0.31	-0.03	0.20	-0.21	-0.03	0.03	0.12
T fall	-0.16	-0.17	-0.33	0.31	-0.03	-0.25	-0.12	0.01	0.15	-0.34	-0.06	0.17	-0.26	-0.08	-0.02	0.08
T winter	-0.32	-0.36	-0.42	0.41	-0.12	-0.36	-0.17	0.00	0.26	-0.19	0.14	0.33	-0.06	0.05	0.11	0.12
T spring	-0.25	-0.24	-0.33	0.32	-0.08	-0.39	-0.23	-0.08	0.24	-0.26	0.04	0.26	-0.13	0.06	0.11	0.19
T summer	-0.02	0.01	-0.22	0.19	0.03	-0.16	-0.08	0.02	0.05	-0.45	-0.25	0.01	-0.41	-0.14	-0.12	0.07
Tmax	-0.05	0.02	-0.16	0.13	0.15	0.02	0.01	0.06	-0.06	**-0.65**	-0.17	-0.11	-0.52	-0.10	-0.05	0.17
Tmax fall	-0.01	0.05	-0.16	0.14	0.16	0.06	0.04	0.08	-0.09	**-0.67**	-0.21	-0.14	-0.56	-0.17	-0.10	0.13
Tmax winter	-0.30	-0.24	-0.33	0.28	0.02	-0.14	-0.04	0.07	0.09	-0.43	0.05	0.12	-0.26	0.05	0.09	0.19
Tmax spring	-0.12	-0.03	-0.16	0.13	0.13	-0.07	-0.05	0.01	0.01	**-0.60**	-0.10	-0.06	-0.43	0.00	0.05	0.25
Tmax summer	0.25	0.32	0.03	-0.05	0.28	0.22	0.10	0.07	-0.23	**-0.80**	-0.43	-0.37	**-0.75**	-0.29	-0.22	0.09
Tmin	-0.25	-0.35	-0.43	0.45	-0.18	-0.55	-0.32	-0.13	0.34	0.00	0.10	0.44	0.06	0.03	0.09	0.07
Tmin fall	-0.23	-0.33	-0.43	0.44	-0.16	-0.52	-0.29	-0.11	0.32	-0.03	0.08	0.41	0.02	-0.01	0.06	0.04
Tmin winter	-0.28	-0.41	-0.45	0.48	-0.21	-0.55	-0.31	-0.11	0.38	0.03	0.18	0.48	0.10	0.04	0.13	0.07
Tmin spring	-0.27	-0.36	-0.41	0.44	-0.19	**-0.60**	-0.38	-0.18	0.38	0.03	0.15	0.48	0.12	0.09	0.16	0.12
Tmin summer	-0.20	-0.28	-0.43	0.41	-0.14	-0.48	-0.28	-0.11	0.26	-0.04	-0.01	0.37	-0.01	-0.01	0.02	0.03
Pa	-0.41	-0.21	0.29	-0.23	-0.07	-0.33	-0.24	-0.16	0.35	0.40	**0.64**	0.14	0.57	0.43	0.48	0.20
Ps	-0.24	-0.19	0.12	-0.10	-0.12	-0.38	-0.33	-0.28	0.32	0.39	0.45	0.24	0.54	0.53	0.50	0.36
PET	-0.26	-0.26	-0.39	0.35	-0.13	-0.35	-0.23	-0.08	0.22	-0.31	0.01	0.26	-0.18	0.08	0.11	0.22
AI	-0.34	-0.19	0.29	-0.24	-0.08	-0.23	-0.21	-0.18	0.26	0.45	**0.60**	0.13	**0.60**	0.45	0.47	0.21
Water balance	-0.07	0.06	0.41	-0.36	0.08	0.07	0.02	-0.05	0.01	0.41	0.34	-0.11	0.42	0.19	0.19	-0.03
Rad	0.03	-0.04	-0.27	0.24	-0.04	-0.04	0.14	0.23	0.08	-0.26	-0.26	0.07	-0.32	-0.26	-0.24	-0.12
Thermal oscillation	0.47	**0.63**	0.38	-0.43	0.43	**0.64**	0.34	0.15	-0.53	**-0.79**	-0.56	**-0.74**	**-0.81**	-0.31	-0.32	0.03

Bold lettering indicates significant Pearson correlation coefficients ($P < 0.05$)

Appendix S3. Supplemental methods S1-S2

Methods S1 Further climatic and location information:

To characterize populations and analyze climate-seedlings trait relationships, we used monthly climatic data (mean, maximum, and minimum temperatures and total precipitation) from the E-OBS v10.0 and CRU TS 3.22 (Europe) datasets for the period 1950–2014 (KNMI Climate Explorer dataset: https://climexp.knmi.nl) (Oldenborgh & Burguers, 2005) combined with Hobo U-12 data loggers (Onset, Pocasset, MA, USA) located at the study sites that recorded air temperature and relative humidity for the period 2012-2014. For temperature, we corrected the climatic interpolation for each location through linear correlations using Hobo recorded data (all correlations between regional and Hobo data were highly significant). Monthly surface solar radiation for each location was determined from the FRESCO v6 dataset gridded at 0.5º for the period 2002-2012 (KNMI Climate Explorer). Potential evapotranspiration (PET) was estimated as a function of the mean monthly temperatures, monthly rainfall and the geographical latitude (Thornthwaite 1948). The De Martonne aridity index was estimated as – (Pa/(T+10)) (De Martonne 1926).

Methods S2 Data normalization

In order to avoid finding differences among populations caused by different growth conditions between years (2010 and 2013), we performed the data normalization per year by using the following formula:

$$X' = \frac{X - Xm}{Xsd}$$

where *X*' is the normalized value of a given variable, *X* represents the original value, Xm is the average value for such variable, and X_{SD} is the standard deviation.

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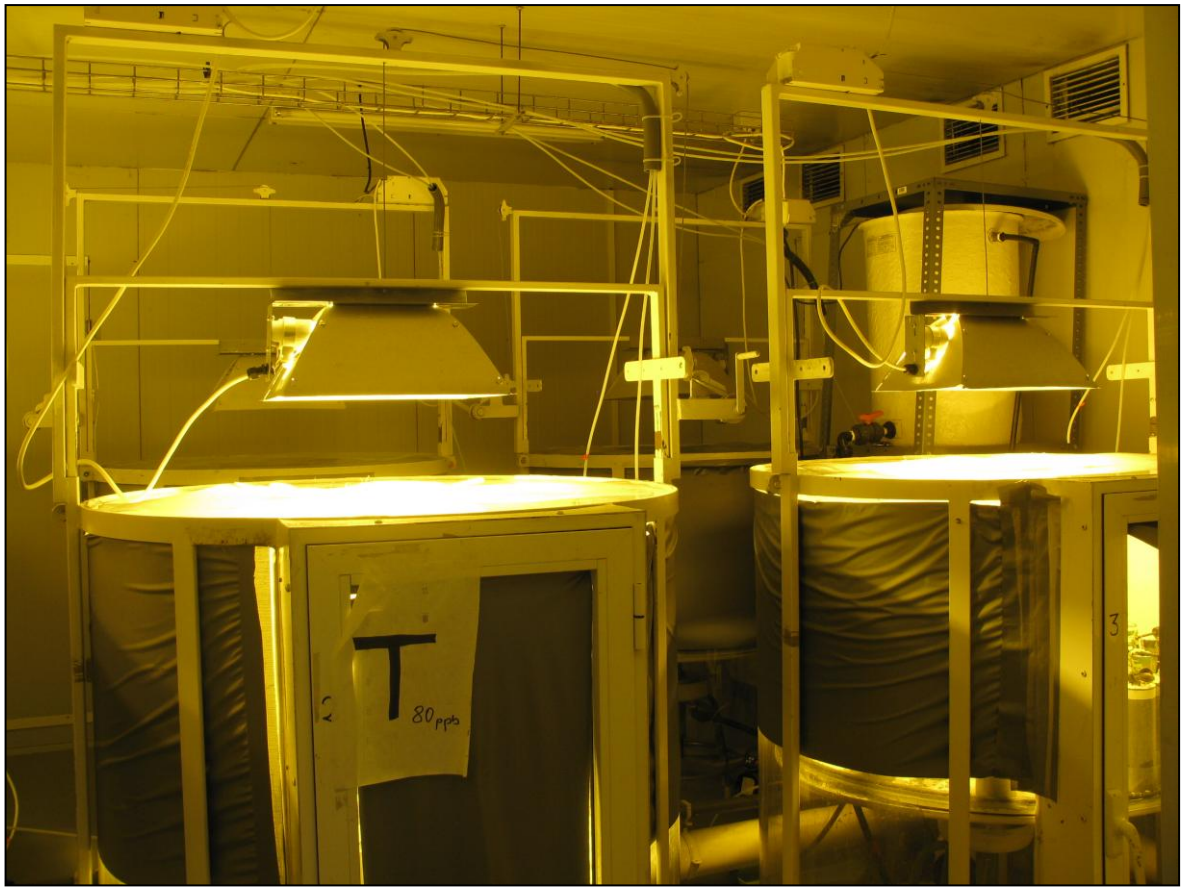
IV. CAPÍTULO 3

Intraspecific sensitivity to oxidative stress: physiological and morphological response to ozone across the latitudinal gradient of the evergreen oak *Quercus ilex*.



Intraspecific sensitivity to oxidative stress: physiological and morphological response to ozone across the latitudinal gradient of the evergreen oak *Quercus ilex*.

Ana García-Nogales, José I. Seco, Juan C. Linares, José Merino

1. ABSTRACT

Tree species subjected to Mediterranean climate undergo high levels of oxidative stress as a consequence of high sun-light radiation, extreme temperatures, water shortage or air pollution. Despite recent insights on the species response to these oxidative stressors, intra-specific variability in plant sensitivity to air pollution is still poorly understood. Over the past decades, alongside oxidative stressors of natural origin, plants have also been affected by anthropogenic factors, such as increased tropospheric ozone (O_3). Therefore, it is mandatory to increase our knowledge on intra-specific adaptive capacity processes in relation to oxidative stress in trees. We investigate the sensitivity of physiological and morphological traits in seedlings of 12 Holm oak (*Quercus ilex*) populations, covering the species latitudinal range, in response to O_3. Seedlings were grown from acorns under controlled ozone treatments lasting three months. The treatment impaired plant physiology to a large extent than morphology. O_3 decreased both photosynthetic rate (39%), actual quantum yield of PSII photochemistry (50%), and increased non-photochemical quenching (98%). The Holm oak populations showed high heterogeneity on the sensitivity to ozone stress, where those native to the range edges displayed the lowest reduction of the photochemical performance. The tolerance of bordering populations seems to be related to their inherent lower stomatal conductance, as well as putative higher constitutive defensive endowments. Our results suggest that both the occurrence of phenotypic canalization and the defence

mechanisms to oxidative stress related to range edge conditions (low temperatures at northern edge, drought at southern edge) may induce resistance to other stressors as O_3.

Key-words: air pollution, range margin pattern, chlorophyll *a* fluorescence, ecophysiology, gas exchange, local adaptation, Mediterranean evergreen oak, phenotypic canalization, phenotypic plasticity, stress tolerance.

2. INTRODUCTION

Mediterranean climate induces high levels of oxidative stress on vegetation as a consequence of high sun-light radiation, extreme temperatures, and/or water shortage (Bussotti, 2008; Flexas *et al.*, 2014). Most of the researches aimed to study the intra-specific response to oxidative stress in Mediterranean trees support a significant heterogeneity among populations. Furthermore, it is reported that such variability is related to the habitat of origin, for instance as a result of low temperatures (Aranda *et al.*, 2005; Cavender-Bares, 2007; Ramírez-Valiente *et al.*, 2015), drought (Gratani *et al.*, 2003; Sanchez-Vilas & Retuerto, 2007; Aranda *et al.*, 2010) or light excess (Balaguer *et al.*, 2001; Sustani *et al.*, 2014; Corcuera & Notivol, 2015); providing evidence of morphophysiological adaptive capacity (García *et al.*, 1998, Balaguer *et al.*, 2001; see Bussotti, 2008 for a review).

Over the past decades, besides oxidative stresses of natural origin, plants have also been affected by anthropogenic factors, such as air pollution-related to rising tropospheric ozone (O_3) concentrations (Krupa, 2000). Even though O_3 is considered the most oxidizing and phytotoxic air pollutant (Ashmore, 2005; Pellegrini *et al.*,

2011a), the investigations on tree species sensitivity to O_3 has commonly neglected the role of the intra-specific variability in response to this stress (Heath & Taylor, 1997; Paoletti, 2006; Calatayud, 2007; Bussotti *et al.,* 2011; but see Paludan-Müller *et al.*, 1999; Minnocci *et al.*, 1999; Elvira *et al.*, 2004). Nonetheless, since phenotypic variation relies on population-level adaptive processes, this knowledge is essential for understand the importance of the evolutionary potential of populations as a source of versatility to cope with environmental stress and to enhance plant survival (Hoffman & Sgrò, 2011).

Leaves are directly affected by O_3, the photosynthetic apparatus being quite sensitive to this pollutant because of its both physical-chemical dynamism and complexity (Fiscus *et al.,* 2005). The impairment of photosynthetic apparatus results in a decrease of metabolic energy available for leaves, stem and roots; which hinders the plant nutrition function, resulting also affected the plant growth (Paoletti, 2006). Plant sensitivity to O_3 is related to both the capacity to activate the avoiding mechanisms (e.g. stomatal closure in order to reduce the ozone uptake) and the repair and compensation processes (Bussotti *et al.,* 2007). The active mechanisms of defence includes an enhancement of the xanthophyll cycle (Pellegrini *et al.,* 2014), increase of maintenance respiration rate (Amthor, 1988), antioxidant and detoxifying enzymes production (Di Baccio *et al.,* 2008), changes in foliar morphology (Bussotti, 2008), thickening of the mesophyll cell walls (Bussotti *et al.,* 2005), among others (Bussotti *et al.,* 2007).

Ozone is a secondary pollutant produced by photochemical reactions of UV radiation, nitrogen oxides and volatile organic compounds (VOCs). These factors are common in the Mediterranean-type ecosystems (Paoletti, 2006). Accordingly, effects of ozone on the general physiological performance and morphology of sclerophyllous Mediterranean species have been reported previously (Bussotti & Gerosa, 2001; Nali *et*

al., 2004; Paoletti, 2006). As regards tree species sensitivity, these works suggested that Mediterranean trees are not threatened by rising ozone concentrations, because of their high amount of defensive endowments, sclerophyllous leaves and their strong stomatal control (Ashmore, 2005).

Holm oak (*Quercus ilex* L.) is an evergreen tree broadly distributed around the Mediterranean Basin. The wide environmental range of Holm oak provides a suitable experimental system to investigate differential adaptive capacity to stress in tree physiology and morphology linked to their habitat of origin (Gratani *et al.,* 2003; Sanchez-Vilas & Retuerto, 2007; García-Nogales *et al.,* 2016). Despite *Q. ilex* sensitivity to ozone stress have been previously assessed (Manes *et al.,* 1998; Ribas *et al.,* 2005a; Vitale *et al.,* 2008; Calatayud *et al.,* 2011; Alonso *et al.,* 2014), biogeographical intra-specific patterns are poorly understood. We assumed that *Q. ilex* individuals from different provenances won't display local adaptations to ozone since such pollutant is homogeneously distributed around the western Mediterranean not-urban areas (Paoletti, 2006). So, O_3 could be used as an unspecific stress factor suitable to gain a better understanding on constitutive generic tolerance to oxidative stress.

As regards the strong dependence of O_3 uptake on stomatal conductance (Reich, 1987; Kerstiens and Lendzian, 1989), the constitutive differential stomatal conductance of each population should result in different ozone uptake. Specifically, we hypothesise that the southern populations, usually subjected to drought, should reflect lower ozone uptake and so, less foliar injury. Alternatively, since foliar injury caused by ozone also depends on plant anti-oxidant defences (Fredericksen *et al.,* 1996; Sandermann *et al.,* 1997), which are usually present in stress adapted plants (Alonso *et al.,* 2001; Tattini *et al.,* 2005; Paoletti, 2006; Laureano *et al.,* 2016); we hypothesise that individuals from

the edges of the species range (both the southern and the northern populations) could display a constitutively high tolerance to a generic stress, as ozone.

The purpose of this work is to analyse the intraspecific tolerance to ozone in *Q. ilex* seedlings native to a wide latitudinal gradient, as well as to assess the impact of ozone on this species. Specifically, we addressed the following questions: 1) How *Q. ilex* physiology and morphology respond to ozone stress? 2) Are there differences in the physiological and morphological response to ozone among *Q. ilex* provenances? 3) Are the provenance differences related to either a climatic or a range margin (edge *vs.* core locations) pattern?

3. MATERIAL AND METHODS

3.1. Study sites, seed collection and culture

Acorns were collected from 12 different populations of *Quercus ilex* L. located along its latitudinal gradient in the western side of its distribution range (Fig. 1). The latitudinal gradient covers core and edge populations throughout a wide range of climatic conditions. Following De Martonne's aridity index (De Martonne 1926) and Potential Evapotranspiration values, populations ranged from humid (Cambo; France, ~43ºN, the northernmost population) to arid (Agadir; Morocco, ~30ºN, the southernmost population; Table 1). Local climate data for each population were obtained from KNMI Climate Explorer (https://climexp.knmi.nl; Oldenborgh & Burguers, 2005) combined with Hobo U-12 data loggers (Onset, Pocasset, MA) located in the field study sites.

The analyses in the 12 populations took place in two different non correlative years. Acorns harvesting was carried out during the autumns of 2010 and 2013 (for

eastern locations and for western locations, respectively). A mean of 1000 acorns were collected per population from 20 randomly assigned trees enough separated to reduce the chance of sampling related trees.

Table 1. Origin of the 12 provenances of *Q. ilex* characterised by latitude, longitude, elevation, as well as mean annual temperature (T), mean maximum temperature in summer (Tmax), mean minimum temperature in winter (Tmin), annual rainfall (Pa), corrected potential evapotranspiration (PET), De Martonne's aridity index, -(Pa/(Tm+10)) and mean solar radiation (Rad).

Code	***Population***	***Country***	***Latitude (N)***	***Longitude (E-W)***	***Elevation (m.a.s.l.)***	***T (ºC)***	***Tmax (ºC)***	***Tmin (ºC)***	***Pa (mm)***	***PET (mm)***	***De Martonne AI***	***Rad (W m^{-2})***
1	Cambo	France	43º 58'	3º 48' E	610	10.9	20.6	2.4	658	*653*	-31.5	508
2	León	Spain	42° 27'	5° 58' W	871	10.8	29.7	-0.5	505	654	-24.3	566
3	Montblanc	Spain	41º 20'	1º 07' E	882	11.3	24.3	2.8	532	*662*	-25.0	559
4	Salamanca	Spain	41° 11'	5° 30' W	886	10.8	25.2	0.7	403	653	-19.4	580
5	Atzeneta	Spain	40º 15'	0º 14' W	970	11.6	23.3	2.2	499	669	-23.1	560
6	Font Roja	Spain	38º 39'	0º 32' W	1270	14.0	30.2	2.6	420	748	-17.5	575
7	Sevilla	Spain	37° 59'	5° 57' W	347	17.1	33.5	5.7	574	890	-21.2	622
8	Alhamilla	Spain	36º 59'	2º 23' W	1370	14.5	30.0	5.1	239	767	-9.8	625
9	Felix	Spain	36º 54'	2º 40' W	1138	13.3	30.5	0.5	310	*730*	-13.3	610
10	Chaouen	Morocco	35° 05'	5° 05' W	939	13.4	25.1	4.7	647	701	-27.6	604
11	Beni Mellal	Morocco	32° 23'	6° 12' W	850	17.2	27.1	8.8	443	*849*	-16.3	681
12	Agadir	Morocco	30° 45'	8° 44' W	1553	21.3	33.5	10.6	309	*1136*	-9.9	695

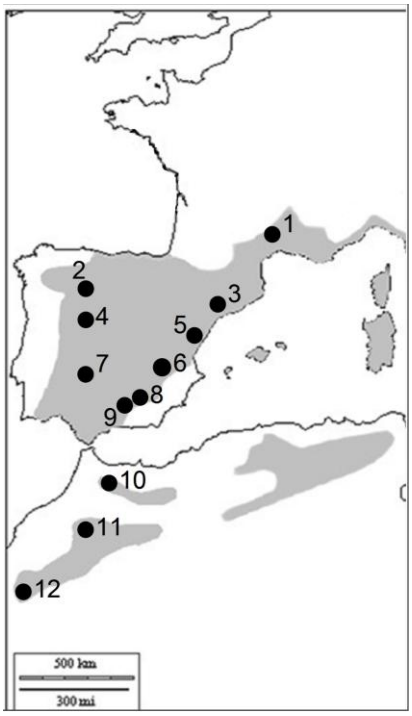


Figure 1. Locations of the 12 sampled populations in the western part of *Q. ilex* distribution range (map modified from Delzon *et al*., 2013). Numbers denoting each population are detailed in Table 1.

Acorns were labelled and placed in trays with perlite substrate for germination under greenhouse conditions at Pablo de Olavide University, Seville (37°21'3N, 5°56'7W, 15 m.a.s.l.). One month after germination, around 100 seedlings per population were randomly selected and sown in 1–litre PVC pots with a 3:1 sand: perlite mixture. The seedlings were kept growing in a controlled-environment greenhouse (at a day/night mean temperature of 19/11ºC, a mean RH of 62%, and a mean photon flux density of 300 µmol $m^{-2}s^{-1}$) for two months.

3.2. Growth conditions and ozone fumigation

When seedlings were roughly 10 cm tall, a mean of 30 plants were randomly chosen per population. Selected seedlings were pooled in two groups, Control and Treatment (15 individuals per group), and were placed in six 1 m^3 growth chambers under the following conditions: day/night temperature of 25/20ºC, day/night RH of 56/80%, photosynthetic photon flux density (PPDF) of 400 µmol $m^{-2}s^{-1}$ at plant height, and 11-h photoperiod. Twice daily plants were watered with tap water using a droplet system. Twice weekly *Hoagland* nutritive solution was added to offset the lack of nutrients in the substrate. To minimize the chamber effect, plants were rotated both inside chamber and between chambers (once weekly).

Ozone treatment consisted in 5 hours (9:00-14:00) per day of fumigation at 80 ± 10 ppb of concentration matching with the light period. Ozone was generated from oxygen using a high-voltage electrical discharge generator (SIR S.A., Madrid, Spain). Air quality inside the chambers was continuously monitored with an ozone analyser (Dasibi 1008-RS, Environmental Corp., Glendale, CA, USA), and ozone concentration was adjusted manually. The fumigation experiment lasted 90 days, so the AOT40 was

roughly 18,000 ppb at Treatment chambers; while in Control chambers it was always low and never exceeded the threshold of 40 ppb (AOT40).

3.3. Gas exchange and Chl a fluorescence measurements

After 90 days of treatment, the physiological measurements were carried out on attached leaves of all plants. One fully-developed leaf was selected from the bottom of the seedling for gas exchange measurements, while one from the top third of the plant was chosen for chlorophyll *a* fluorescence determinations (n = approximately 15 leaves per population in both analyses). Selected leaves had already emerged before fumigation started in growth chambers.

Gas exchange measurements were made in attached leaves (one leaf per seedling) using an open infra-red gas exchange system (Model CIRAS-I, PP-Systems, Edinburgh UK). All the determinations were performed at ambient CO_2 concentration (390-400 ppm), 25ºC, 60% RH, and 450-500 µmol $m^{-2}s^{-1}$ PPFD.

Inside growth chambers, modulated chlorophyll *a* fluorescence measurements were carried out with pulso-modulated portable fluorometer (FMS 2, Hansatech Instruments Ltd, Norfolk UK). For light measurements, we adapted selected leaves to a PPFD of 450 µmol $m^{-2}s^{-1}$ for one hour to ensure all samples were measured at the same conditions. Subsequently, the sampled leaf was dark adapted for 30 minutes in order to the primary quinone acceptor of Photosystem II (Q_A) becomes maximally oxidized. Light and dark measurement protocol is described in Supporting Information Appendix S1, Methods S1.

3.4. Biomass and structure

At the end of the experiment, control and treated seedlings were separated into stem, leaf and root fractions, and oven dried at 70ºC until constant weight. Previously, three fresh leaves per seedling were collected to measure mean leaf mass (LW) and mean leaf-blade size (LS). SLA (LS to LW) and S/R (shoot to root weight) were calculated for each seedling. Total leaf area per seedling (LA) was calculated by weighing the total number of leaves and multiplying by its LS. For each seedling, LAR (total leaf-area to plant weight) was calculated by dividing its total leaf area by its total seedling weight; while LWR (total leaf weight to plant weight) was estimated by dividing the total leaf weight by the total seedling weight.

3.5. Statistical analyses

The differences between mean gas exchange and fluorescence values for control and treated plants for each population, as well the effect of *Year*, were analysed using a non-parametric test for mean comparison (U-Mann Withney test). In order to test the effect of *Population*, a non-parametric multiple test (Kruskal-Wallis test) was applied.

As differences were observed between years (Table 2), physiological and morphological variables were relativized (i.e. expressed as treated/control ratio for each population) and normalized (see Appendix S1, Methods S2) in order to display different sensibility to ozone among populations (Table S1 and S2 in Appendix S2 to see the original values). Thereby, we avoid finding differences between populations caused by different conditions between years. The relative effect of ozone on gas exchange, fluorescence and biomass variables were assessed by a factorial ANOVA for each different year to analyse the effect of *Ozone*, *Population* and their interaction. Also a one-way ANOVA was performed in the normalized values to analyse the relative effect

of ozone considering all populations. Data were transformed when was necessary to meet the assumptions of ANOVA; and when differences were significant, *a posteriori* test were performed (Tukey's *post hoc* HSD) to make individual comparisons at the population level. A principal component analysis (PCA) was conducted to summarize the ozone effects (normalized Treatment/Control variables for each population) on physiological and morphological traits. The results were analysed using statistical software STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA). We performed linear regressions to test whether ozone sensitivity (normalized effect of ozone on physiological and morphological variables) was associated with climate of origin and/or with population particular traits (measured in Control plants at 90 days of experiment). Besides, regression analyses were used also to find relationships between relative variations in physiological and morphological traits of each population with latitude (geographic location).

4. RESULTS

4.1. Ozone effects on gas exchange, chlorophyll a fluorescence and seedlings structure

Several external signs of injury were observed in leaves of the fumigated plants (chlorosis, yellowing, black spots, mottle stipple bronzing; Fig. S1 in Appendix S3, Supporting Information). The effect of ozone treatment on the variables considered was qualitatively general for the 12 populations (Tables S1-S3 in Appendix S2) and resulted in significant changes in almost all the variables studied, excepting F_o and LA (Table 2). In addition, the treatment effects were quantitatively different depending on both the

population and the year of experiment considered (see Table 2 and Tables S1-S2 in Appendix S2).

As a result of 90 days of treatment, both stomatal conductance (*GS*) and photosynthetic rate (*PN*) decreased significantly (–22.0 ± 2.1% and –39.1 ± 1.7% respectively, $P < 0.001$; Table 2). In the photosynthesis-conductance relationship, the ozone treated seedlings displayed lower correlation compared with control ones (Fig. 2a). Besides, a significant increase in the intercellular CO_2 concentration (*Ci*) was noted at the end of the experiment (+36.9 ± 3.1%, $P < 0.001$) in treated plants compared with Control. A significant decrease in the water use efficiency (*WUEi*), expressed as *PN/GS*, was observed (–20.8 ± 2.3%, $P < 0.001$) in the treated seedlings (Table 2).

Considering the overall populations' response in relation to chlorophyll *a* fluorescence parameters, the fumigated plants showed a significant decrease in the maximum quantum yield of Photosystem II (PSII) photochemistry in the dark-adapted state (F_v/F_m) compared with control ones (–4.1 ± 0.3%, $P < 0.001$; Table 2); indicating that ozone impaired the efficiency of PSII. This reduction was attributable to a decrease in F_m in treated plants compared with Control (–13.3 ± 1.2%; $P < 0.001$), since F_o remained similar to Control at the end of the experiment (Table 2).

Table 2. . Average ozone effects relative Control to Treatment (%) in the main variables studied in the twelve *Q. ilex* populations considered all together at 90 days of experiment. Asteriks denote significant effects of ozone, population and year. $P < 0.05$*, $P < 0.01$**, $P < 0.001$***.

Variables	***Ozone effects (%)***	***Significant effects***		
		O_3	**Pop**	**Year**
PN (µmol CO_2 cm^2g^{-1})	−39.1 ± 1.7	***	***	***
Gs (mmol H_2O cm^2g^{-1})	−22.0 ± 2.1	***	***	***
Ci (µmol mol^{-1})	36.9 ± 3.1	***	***	***
WUE (µmol $mmol^{-1}$)	−20.8 ± 2.3	***	***	***
Fo	0.01 ± 1.14	n.s.	***	***
Fm	−13.3 ± 1.2	***	n.s.	n.s.
Fv	−16.4 ± 1.3	***	n.s.	n.s.
Fv/Fm	−4.1 ± 0.3	***	***	***
ΦPSII	−26.9 ± 1.5	***	***	***
Fs	−29.1 ± 1.6	***	***	***
Fm'	−35.9 ± 1.7	***	***	***
Fq'	−50.2 ± 2.1	***	***	***
qP	−13.3 ± 1.3	***	***	n.s.
NPQ	97.9 ± 6.9	***	***	***
(1-qP)/NPQ	-42.6 ± 2.8	***	***	***
%D	40.5 ± 2.2	***	***	***
%X	-7.6 ± 1.1	***	***	***
Leaves	−10.1 ± 2.9	n.s.	***	***
Shoot (g)	−14.7 ± 2.7	**	***	**
Roots (g)	−26.1 ± 2.3	***	***	***
Total mass (g)	−20.1 ± 2.2	***	***	***
LS (cm^2)	−4.4 ± 2.0	n.s.	***	n.s.
LW (g)	0.3 ± 2.2	n.s.	***	**
SLA (cm^2g^{-1})	−3.0 ± 0.8	*	***	n.s.
LA (cm^2)	−10.0 ± 2.7	n.s.	***	n.s.
S/R (gg^{-1})	20.3 ± 3.4	***	***	n.s.
LAR (cm^2g^{-1})	11.1 ± 1.9	**	***	n.s.
LWR (gg^{-1})	12.6 ± 1.7	***	***	n.s.
n	211			

Referring to fluorescence in light conditions, non-photochemical quenching (*NPQ*), that represent the amount of energy absorbed dissipated as heat in plants, increased in treated seedlings of all populations after 90 days of treatment (+97.9 ± 6.9%, $P < 0.001$; Table 2) compared with Control. The combined effect of F_m decrease

(less light energy absorbed) and *NPQ* increase (more energy dissipated) resulted in a significant decrease of F_m' values (–35.9% ± 1.6, $P < 0.001$), leading to a less amount of energy that potentially would be targeted to the electronic transport chain. The fraction of opened PSII reaction centres (q_P) experienced a significant decrease in the fumigated plants regarding to control ones (–13.3 ± 1.3%, $P < 0.001$), which means a less effective reoxidation of the primary electron acceptor (Q_A) caused by ozone. The decline in F_m' and q_P values resulted in a decrease in both the amount of energy leading to the electron transport chain (F_q') and the actual quantum yield of PSII photochemistry (Φ_{PSII}) in O_3-treated plants in comparison with control ones (–50.2 ± 2.1% and –26.9 ± 1.5%, respectively, $P < 0.001$; Table 2). Fig. 2b displays the relationship between *PN* and F_q' for both treated and control seedlings: the higher correlation observed in the Treatment, compared with the Control, suggests that ozone induces a decrease in electrons required to fix carbon. This suggests a regulation in activity of photosynthetic enzymes to use more efficiently the photochemical energy in presence of a stress factor, such as ozone.

The effect of the O_3 treatment on seedling biomass was general for all the populations and resulted in a significant decrease of both the total mass (–20.1 ± 2.2%, $P < 0.001$) and that of the different organs studied (Table 2). The root biomass showed a stronger decrease than the observed in above-ground fraction (–26.1 ± 2.3% vs. –14.7 ± 2.7%, respectively, $P < 0.01$) in the treated seedlings regarding to control ones. Consequently, the treatment resulted in a significant increase of the biomass allocation indices studied (S/R, LWR, LAR) of +20.3%, +11.1% and +12.6%, respectively ($P < 0.01$) owing to the lower decrease of above-ground fraction compared with below-ground. Besides, SLA underwent a significant decrease (–3%, $P < 0.05$; Table 2).

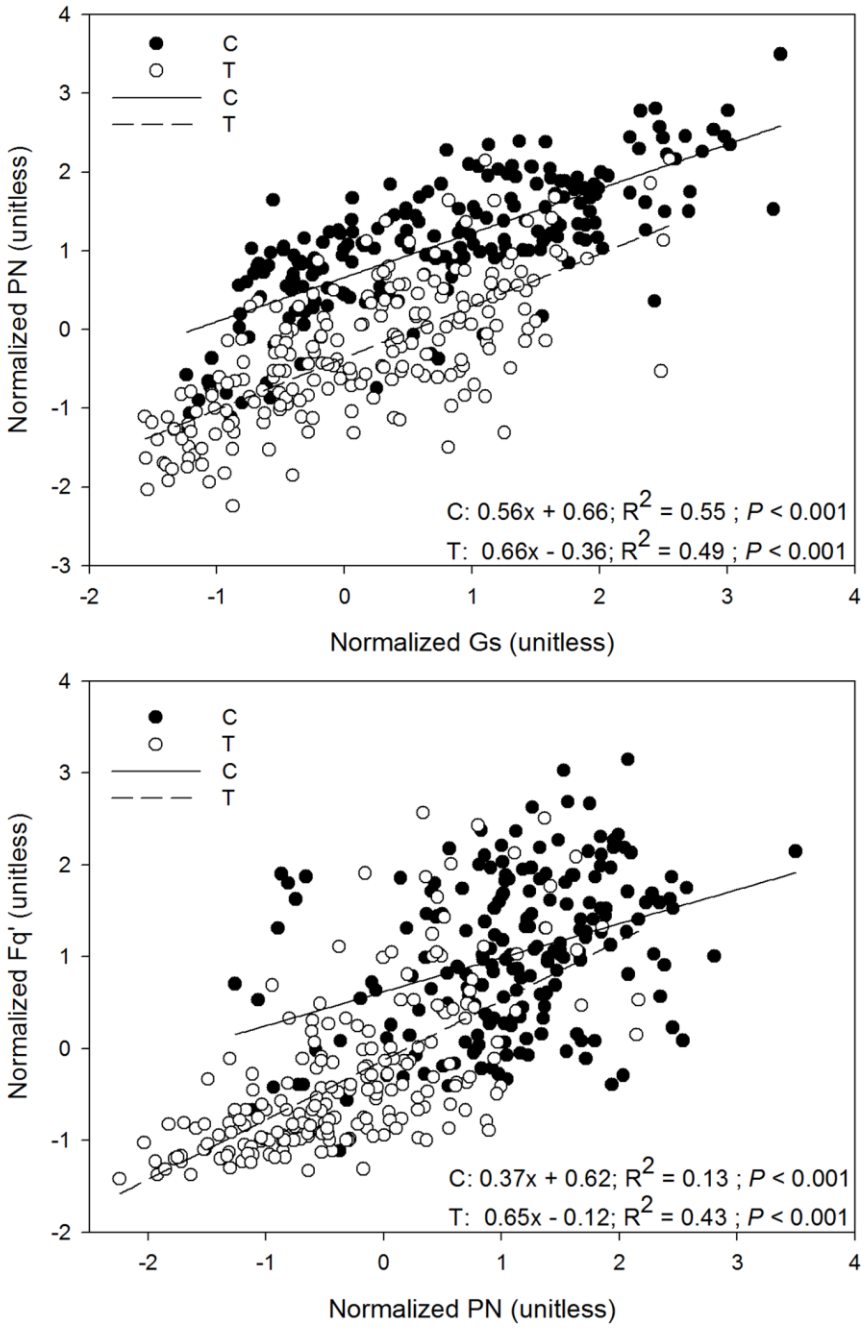


Figure 2. Linear regressions of normalized net photosynthetic rate (*PN*) against normalized stomatal conductance (*GS*) (a); and normalized photochemical quenching ($F_{q'}$) against normalized *PN* (b) in leaves of Treatment (n = 208) and Control (n = 203) of *Quercus ilex*. Regression lines, Pearson's correlation coefficient (R^2) and *P*-value are given for each treatment.

4.2. Relationships between ozone sensitivity and climate and population traits

Correlations between the normalized variation (i.e. mean loss or increases normalized values) of physiological variables (and total mass) of seedlings as a consequence of the treatment and the characteristic traits of each population (i.e. mean Control normalized values), appear in the Table 3. The differential response to treatment among populations was the result of differences in the mean *GS* values of control seedlings, like showed the significant positive relationships with the relative loss of *PN* and F_v/F_m of each population. Besides, as a result of the treatment, significant negative correlations were observed between the S/R averaged Control values of each population and its relative loss in *PN*, F_v/F_m, Φ_{PSII}, F_q', and its relative increase in *%D*. In turn, the mean SLA values of control seedlings showed significant negative relationships with the *PN*, *GS*, Φ_{PSII} and F_q' reductions. On the other hand, no significant correlation was observed between the relative variations of variables considered in response to ozone and mean climatic values at population origin.

4.3. Intraspecific response to Ozone exposure

The variation induced by ozone among populations in the normalized physiological and morphological variables is represented in Figs. S2, S3 and S4 in Appendix S3. In our study, significant among population differences were observed in gas exchange and photochemical variables. On the other hand, the effect of treatment in structural and allocation biomass variables did not show interpopulation variability, with the exception of LAR and SLA ($P < 0.05$; Fig. S3 in Appendix S3). This exception was related to the Felix population, which presented the highest reduction in leaf area, leading to the highest decreases in SLA and LAR values.

Table 3. Pearson correlation coefficients between mean variation of the normalized variables of Treatment and mean climatic values/mean Control values per population at 90 days of experiment. Significant correlations ($P < 0.05$) are noted in bold.

	Variation										
	PN	*GS*	*Fv/Fm*	Φ_{PSII}	*Fq'*	*qP*	*NPQ*	(1-*qP*)/*NPQ*	*%D*	*%X*	Wtotal
GS	**0.59**	0.29	**0.77**	0.52	0.52	0.41	–0.07	0.11	0.42	0.03	–0.10
Wtotal	–0.29	–0.02	–0.08	–0.17	–0.15	–0.17	–0.02	0.02	–0.04	0.08	–0.10
S/R	–**0.78**	–0.44	–**0.68**	–**0.78**	–**0.82**	–0.47	–0.45	0.43	–**0.77**	0.02	–0.22
SLA	–**0.77**	–**0.59**	–0.52	–**0.59**	–**0.61**	–0.37	–0.24	0.13	–0.53	0.06	–0.26
LA	–**0.65**	–0.36	–0.41	–0.53	–0.50	–0.42	–0.20	0.14	–0.40	0.02	–0.16
T	0.13	0.24	-0.11	-0.02	-0.25	0.07	–0.28	0.30	–0.35	0.11	0.04
Tmax	0.39	0.23	0.19	0.17	0.09	0.16	–0.08	0.13	–0.02	–0.05	-0.29
Tmin	0.01	0.13	-0.11	-0.09	-0.31	-0.03	–0.30	0.26	–0.36	0.06	0.30
Pa	-0.42	-0.56	-0.03	0.03	-0.02	0.18	–0.27	0.07	–0.15	0.35	0.31
PET	0.13	0.27	-0.18	-0.17	-0.40	-0.05	–0.31	0.35	–0.44	0.09	0.02
AI	-0.43	-0.54	-0.07	-0.08	-0.04	0.03	–0.16	–0.01	–0.08	0.25	0.26
Rad	0.20	0.39	0.01	0.11	-0.05	0.12	–0.10	0.16	–0.14	0,04	0.02

The results of PCA (Principal Component Analysis) are displayed in Fig. 3 and Table S4 in Appendix S2. We only show the results of PCA for the physiological variables owing to the lack of differences among populations in response to the treatment in the morphological traits. The first factor absorbed 37% of the total variation and includes negative relationships with most of the variables expressing PSII performance (F_v/F_m, F_q', F_v'/F_m', Φ_{PSII}, *%P*), as well as a small contribution of Photosynthetic rate (*PN*); and positive relationships with variables related to energy dissipation (*NPQ*, *%D*). The second factor absorbed 18% of variance and includes negative relationships with q_P, F_o and F_m, and positive relationships with (1- q_P)/*NPQ* and *%X*.

Considering both factors jointly, populations were grouped into three different groups (Fig. 3): the first group was in the left bottom position and included Cambo and

Agadir populations; the second group was located in the right bottom position and included Alhamilla, Salamanca, Chaouen and Font Roja; and finally, the third group was above zero in Axis 2 and includes populations from León, Beni Mellal, Sevilla, Atzeneta, Felix and Montblanc.

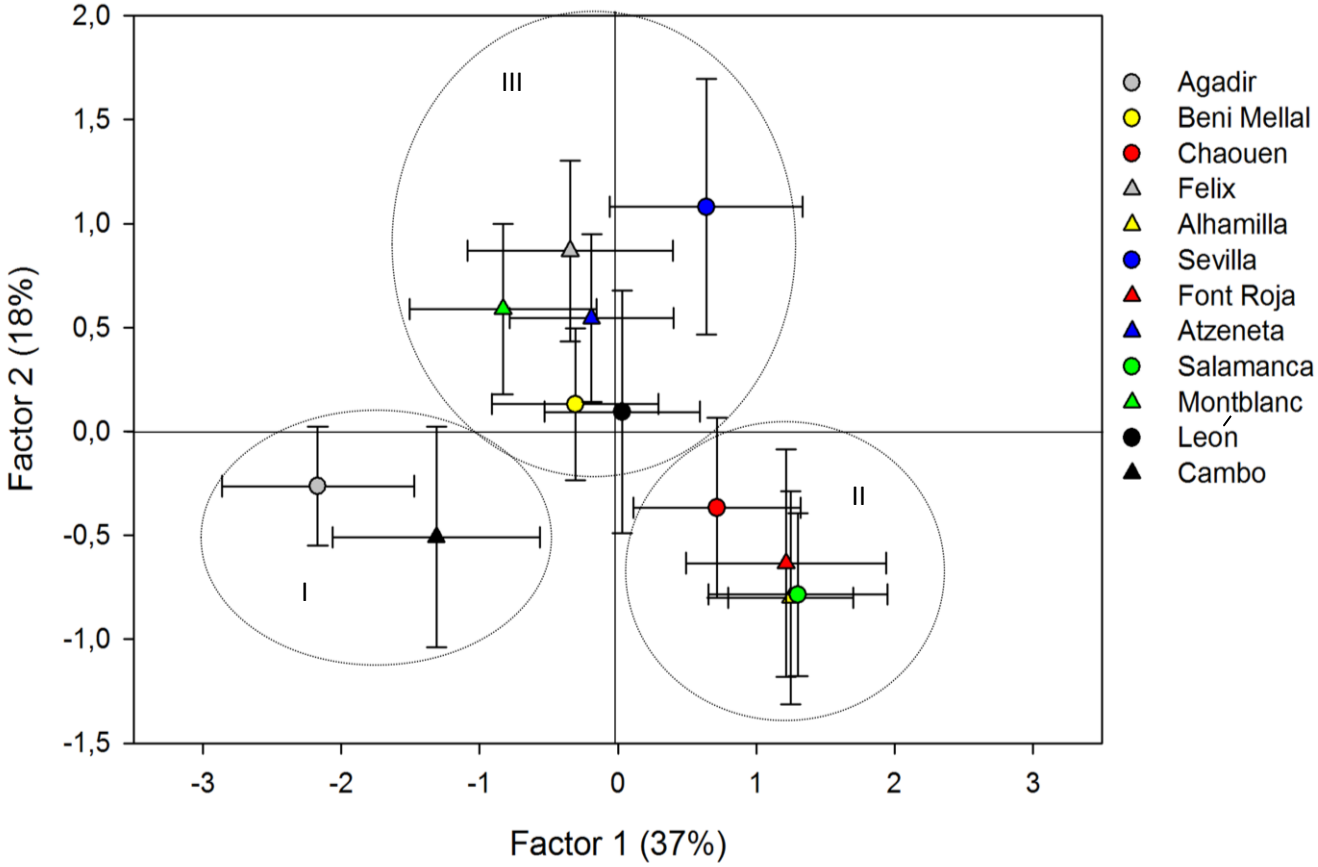


Figure 3. Results of the PCA showing the mean values (± SE) of the first and second factor coordinates for the ozone effect on each population. The percentage of variance accounted by each factor is noted.

The most O_3-resistant populations, in terms of photochemical efficiency, were those from the first PCA group. The seedlings from the northernmost and the southernmost populations (Cambo and Agadir, respectively) showed a reduction below the average of the rest of populations in F_v/F_m, $\Phi_{PSII,}$ F_q' and *%P* (Figs. 5 and S4 in Appendix S3). Moreover, both populations presented a lower increase in the fraction of

the absorbed energy dissipated as heat *(%D*) compared with the average of the populations. Besides, Cambo showed the lowest reduction of *PN* normalized values, while presented the significant lowest reduction of instantaneous *WUE* (Fig. S2 in Appendix S3).

The treated seedlings from populations included in the second group showed a combination of high increases in heat dissipation (*NPQ*) and high reductions in the photon excess ((1- q_P)/(*NPQ*)), resulting in less photochemical energy flowing through the electron transport chain, and therefore, in a high reduction of F_q' (Fig. S4 in Appendix S3). These high *NPQ* increases were significantly correlated to a higher loss in total weight (R^2 = 0.56, P < 0.01; Fig. 4). In addition, these populations displayed higher values for intercellular CO_2 concentration (*Ci*) compared with the rest of populations (Fig. S2 in Appendix S3). Therefore, this group was considered the most O_3-sensitive populations in terms of both photochemical performance and seedling biomass.

Populations grouped in the third group showed intermediate values of variation in gas exchange and fluorescence variables in response to ozone treatment. The loss in the amount of light energy absorbed (F_m, F_v) in these populations, in general, was above the average regarding to the rest (Fig. S4 in Appendix S3). Given that these populations presented both relative high decreases of the fraction of opened PSII reaction centres (q_P) and minimum increases of *NPQ*, resulted in low reductions of (1–q_P)/(*NPQ*), and therefore, high risk of oxidative stress than the remaining populations.

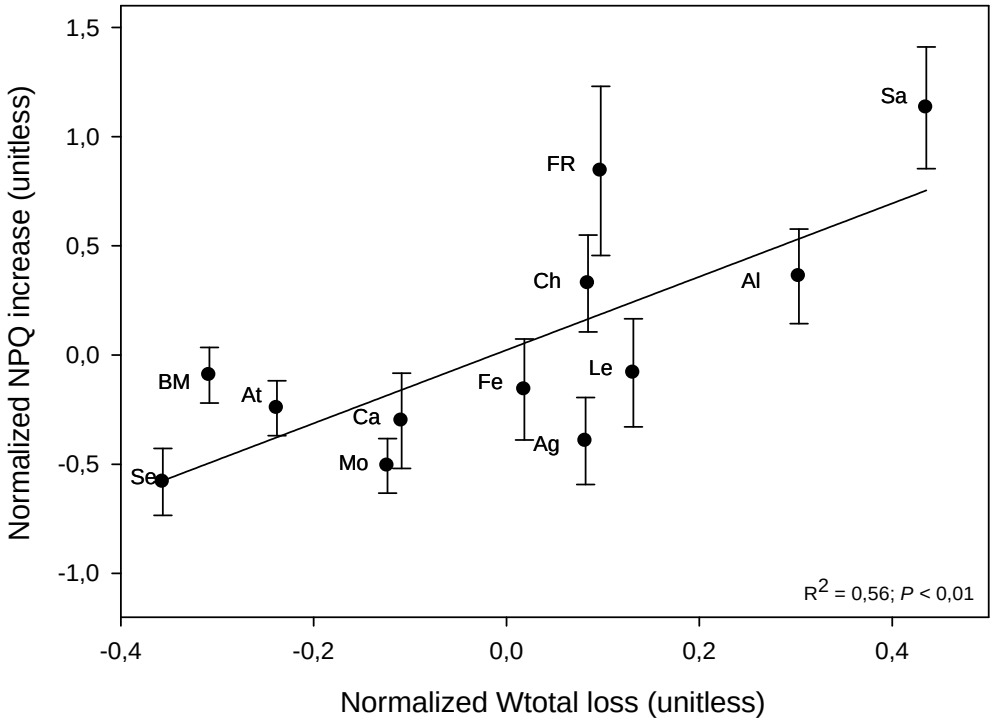


Figure 4. Linear regression of normalized values ozone-variation of both total weight of seedlings and non-photochemical quenching (*NPQ*) at the end of the experiment.

4.4. *Latitudinal trends*

The different sensitivity to ozone observed in the *Q. ilex* populations considered had a latitudinal exhibition. The variables expressing PSII performance, as Φ_{PSII} and F_q' variation, showed highly significant quadratic relationships with latitude (R^2 = 0.74 and R^2 = 0.71, respectively, P < 0.01; Fig. 5), suggesting that the photochemical apparatus of the edge populations was more resistant to ozone stress than the core ones. In addition, other key photochemical variables (F_v/F_m, F_v'/F_m', q_P and *%D*) showed the same trend (Fig. 5).

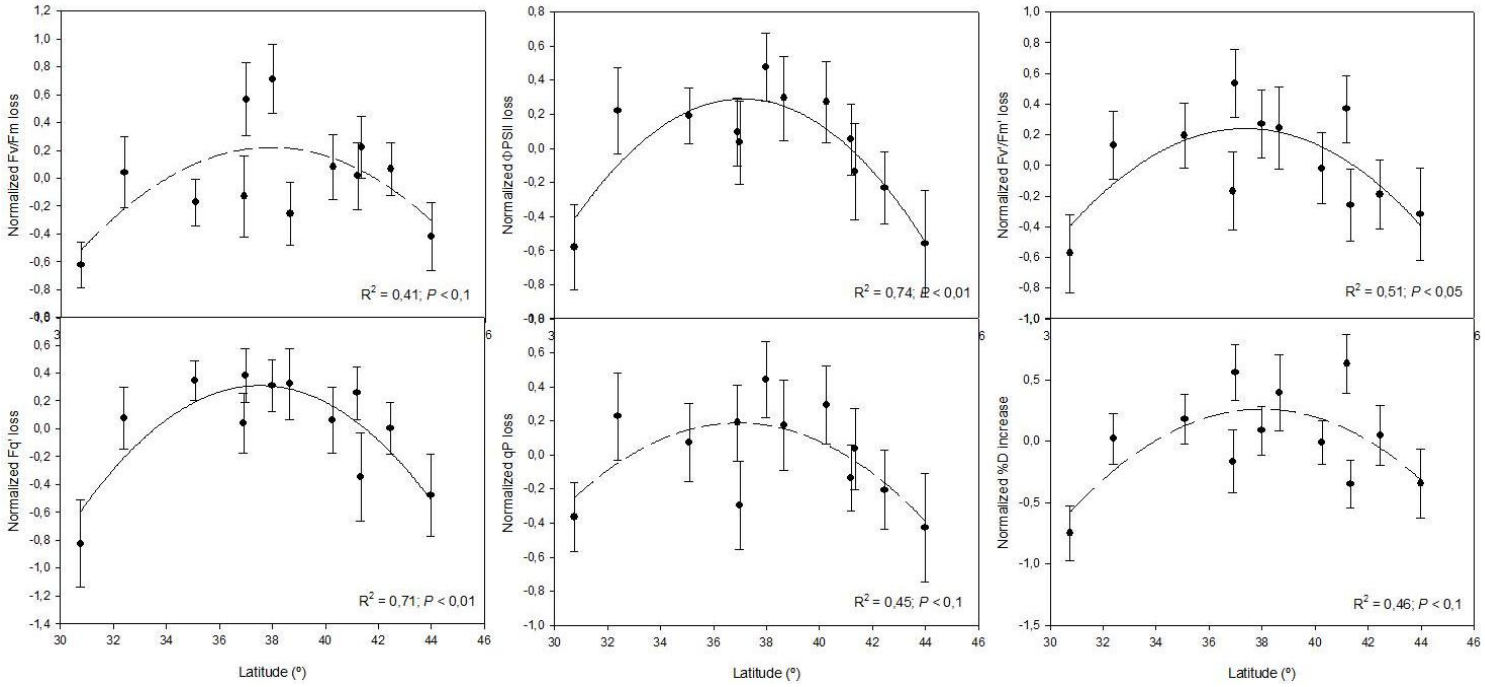


Figure 5. Quadratic relationships between latitude of origin and normalized ozone-variation (losses or increases) data of mean of the main fluorescence variables for each population at 90 days of experiment: maximum quantum yield of PSII photochemistry (F_v/F_m) (a), actual quantum yield of PSII photochemistry (Φ_{PSII}) (b), intrinsic PSII efficiency (F_v'/F_m') (c), photochemical quenching (F_q') (d), coefficient of photochemical quenching (q_P) (e), and fraction of the light absorbed dissipated in the PSII antennae (%*D*) (f).

Note: positive or negative values indicate changes (losses or increases) above or below the year mean value, respectively.

5. DISCUSSION

5.1. Quercus ilex response to ozone stress

Mediterranean vegetation has evolved under conditions of marked oxidative pressure and it is usually considered tolerant towards anthropogenic oxidative stress factors as well (García *et al.*, 1998), such as ozone (Bussotti & Gerosa, 2002; Paoletti, 2006), albeit biogeographical patterns of physiological and morphological intra-specific differences in response to ozone have received less attention.

The ozone exposure throughout 90 days impacted strongly in the physiology and the biomass of *Q. ilex*, whereas the seedling structure was affected to a lesser extent; despite this species has been considered as low sensitive to ozone (Paoletti, 2006; Calatayud *et al.*, 2011). In our case, the ozone fumigation was quite severe, as was confirmed partly by the several visual foliar injuries (Fig. S1 in Appendix S3, Supporting Information). Specifically, ozone stress reduced both net photosynthesis (*PN*) and photochemical efficiency (F_v/F_m, Φ_{PSII} and F_q'), and increased non-photochemical quenching (*NPQ*) in the treated plants regarding to control ones (Table 2). Similar physiological changes (decrease in *PN*, *GS* and F_v/F_m) caused by ozone have been reported previously for Holm oak (Ribas *et al.*, 2005b; Alonso *et al.*, 2014). Some of these studies on *Q. ilex* showed analogous but non–significant ozone-induced effects on gas exchange rates or photochemical properties in comparison with our results (Calatayud *et al.*, 2011; Mereu *et al.*, 2011). This dissimilar response may be probably because the intensity of the treatment was lower in the cited studies.

In global terms, the comparatively higher reduction in *PN* than in stomatal conductance (*GS*) in the fumigated plants (compared with control ones), resulted in the alteration of the slope of the stomatal conductance-photosynthesis curves, in the

decrease in *PN*/*GS* (*WUEi*), as well as in the increase in intercellular CO_2 concentrations (*Ci*) (Table 2, Fig. 2a). These outcomes are coincident with those reported in the literature for chronic ozone exposure (Lombardozzi *et al.,* 2012; Pellegrini, 2014; Pellegrini *et al*., 2015). All together suggests that the reduction in *PN* values was due to injury at mesophyll cells caused by ozone, and, to a lesser extent, stomatal closure.

Regarding the relationship between *PN* and photochemical performance, Bussotti *et al.* (2007) posed that the photochemical efficiency is not necessarily direct related to CO_2 assimilation (*PN*), since the electrons flux (associated with F_q') may be rerouted to other biochemical pathways, such as reduction of O_2, ammonium, nitrite, sulphate or thioredoxin. This could explain the lower correlation between *PN* and F_q' in the control plants compared with the fumigated ones (Fig. 2b). This stronger correlation in the treated plants can be explained by an economic energy/carbon budget optimization at the plant level, similarly as the described by Pellegrini *et al.* (2014) for *Tilia Americana*. These authors postulated that when carbon fixation is low (for instance, under ozone induced stress) the plant should use all the available energy as economically as possible in order to maintain a positive net CO_2 budget.

Overall, ozone treatment induced a significant reduction in variables related to Photosystem II (PSII) performance, such as maximum quantum yield (F_v/F_m), actual PSII efficiency (Φ_{PSII}) or photochemical quenching (F_q') (Table 2). According to literature, a decrease in these variables could be interpreted as fotooxidative damage in PSII reaction centres and photosynthetic pigments losses (Calatayud & Barreno, 2004). In our study, we observe some evidence suggesting the decay in photochemical efficiency caused by fotooxidative damage, such as the impairment at mesophyll cells (described above), together with the lower fraction of PSII reaction centres opened (q_P decrease contributes to reduce electron flux through the electron transport chain) and the

destructive oxidation of photosynthetic pigments (data not shown). On the other hand, some authors have suggested that a reduction in variables relative to PSII performance may result from a down-regulation mechanism associated to a controlled dissipation of the excess of absorbed light (Calatatud & Barreno, 2004; Bussotti *et al*., 2007). Thereby, the PSII apparatus may act as a cooling system reducing the over-excitation risk of PSII. The decrease in *PN* involves a decrease in the CO_2 assimilation capacity, which induces a lower demand of reducing power and energy (NADPH and ATP) (see Calatayud *et al.*, 2010; Pellegrini *et al.*, 2011b; Burkart *et al.*, 2013), and this may lead to a PSII over–reduction (Calatayud & Barreno, 2004). As a result, it would be necessary a reduction in the energy through the electron transport chain that alleviate the over-excitation pressure in PSII. Our results support this argument, since the general increase observed in heat dissipation of the absorbed light energy (*NPQ* and *%D*) suggests that controlled dissipation of the energy at antenna level is occurring. Indeed, the *NPQ* and *%D* increases have been reported as a photoprotective mechanism involving the activation of the xantophyll cycle under stress situations (Demmig-Adams *et al.* 1996), as ozone exposure (Alonso et al 2001). In addition, the observed decrease in the $(1– q_P)/NPQ$ ratio, which is a good estimator of the photon excess (Calatayud & Barreno, 2004), suggests that *NPQ* values were high enough preventing greater damage being caused to PSII. All together indicates that, in seedlings under treatment in our study, not only is occurring fotooxidative damage, probably at the dark phase (the most sensitive part of photosynthesis; Fiscus *et al.*, 2005), but also down-regulation, which prevents the potentially harmful accumulation of excitation energy (ROS). It is important to point out that the basal fluorescence (F_o) values in the treated plants remained similar than those of Control, suggesting that there is not occurring a chronic photoinhibition in the reaction centres (Krause, 1988; Pellegrini *et al.*, 2011b).

According to some studies carried out on different tree species (Ribas *et al.*, 2005b; Calatayud *et al.*, 2007), the effects in physiology cited above resulted in significant reductions of total mass and different structure (LA, S/R, LAR, LWR) in the treated plants, compared with Control. Nevertheless, the morphological traits were affected by ozone to a lesser extent, compared with physiological ones (Table 2), in agreement with other *Quercus* species (Elvira *et al.*, 2004). Besides, it is acknowledged the higher plasticity in physiology compared with morphology in oak species (García *et al.*, 1998, Valladares *et al.* 2002a, Gimeno *et al.* 2009). The differences between the strong treatment-induced decreases in the physiology *vs.* the slighter ones in the morphology could be explained, in part, because the primary target of oxidative stress likely is the Calvin cycle reactions and later the PSII photochemistry (Pellegrini *et al.* 2011b, and 2014). Whatever, ozone seems to cause an immediate response in physiology, while morphology appears to integrate these response in a long-term. Thus, it would be expected that a more durable treatment reflects a greater effects on seedlings morphology than those observed in this study.

Even so, the treated seedlings displayed higher growth reduction for root mass than for shoot mass; resulting in higher S/R than controls. This outcome confirms the observations of other studies suggesting that root growth is one of the most sensitive indicators of chronic ozone stress in plants (Paludan-Müller *et al.*, 1999; Ashmore, 2005). This response may be caused by reductions in available carbon of photosynthesis because of the strong decrease in photosynthetic rate (Table 2), together with an increased carbon demand for above-ground repair and replace mechanisms at the expense of roots (Cooley & Manning, 1987; Paludan-Müller *et al.*, 1999; Andersen, 2003). Besides, other studies have found that ozone reduced phloem translocation

capacity of sugars to either roots or shoots, which results in accumulation of soluble sugars and starch in either shoots or leaves (Lázaro *et al.*, 2013).

SLA values showed a slight significant decrease in response to ozone, owing to leaf weight (LW) kept the same values than Control (Table 2), whereas leaf-blade size (LS) experienced a decrease (non–significant), according to was observed previously by Ribas *et al.* (2005b) for *Q. ilex*. The decrease in SLA observed in our study is a typical response of plants growing under stress. This response could be related to the thickening of the palisade mesophyll cell walls, in order to increase the cells' mechanical resistance, or to increase the detoxifying processes linked to the walls' enzyme activities (Bussotti *et al.*, 2005).

5.2. Interrelationships between ozone sensitivity and population characteristics

No relationship was found between the sources climatic factors and the inherent O_3-resistance, in agreement with Lyons *et al.* (1997). This finding could be explained because the lack of relationship between the normalized *GS* and the local climate conditions reported previously for these populations (Chapter 2). Our results revealed a significant and positive relationship between the mean stomatal aperture of control seedlings and both *PN* and F_v/F_m decreases (Table 3), as has been reported in several studies (Reich, 1987; Calatayud *et al.*, 2007). It has been argued that the most appropriate way to explain plant ozone response is by considering the effective ozone flux (Ashmore, 2005; Matyssek *et al.*, 2007; Paoletti *et al.*, 2008). This approach considers that, in part, the stomatal conductance is one of the characteristics determining a species' resistance to air pollution (Reich & Amundson, 1985), because the real dose (i.e. the amount of pollutant gas reaching in the leaf mesophyll) is a function of the degree of stomatal opening (García *et al.*, 1998). The other part is the defence, i.e., the

capacity of detoxification mechanism to counteract the detrimental effects of the ozone taken up by the plant (Bussotti *et al.*, 2005). In our case, the *GS* among populations explained the 35% and 59% of the *PN* and F_v/F_m reductions, respectively.

A variety of studies conducted in several taxa, including *Q. ilex* (Bussotti *et al.*, 2005; Bassin *et al.*, 2007; Calatayud *et al.*, 2011), have documented that thicker leaves are more ozone-tolerant than thinner leaves: on one hand, thick walls can affect gas diffusion inside the leaves (Paoletti, 2006); and, on the other hand, a high tissue density is considered to be able to better feed detoxification process (Bussotti, 2008). However, contrary to our expectation, significant negative relationships were observed in our study between SLA and *A*, *GS*, Φ_{PSII}, F_q' decreases caused by ozone (Table 3); suggesting that populations with higher SLA (edge populations, among others, Fig. S5a in Appendix S3) are less sensitive to ozone treatment. For its part, significant negative relationship were observed between the inherent S/R and *PN*, F_v/F_m, Φ_{PSII}, F_q' decreases and *%D* increases caused by ozone (Table 3); suggesting that populations with higher aboveground biomass fraction in relation to roots, i.e. higher S/R values (edge populations, Fig. S5b in Appendix S3) appear to be less sensitive to ozone treatment. These results related to SLA and S/R seem to be a paradox, since it would be expected that the larger fraction of structure exposed to the pollutant, the more damage in the photosynthetic apparatus. Perhaps, by investing more energy to aerial parts, which is the energy-gathering fraction of biomass, could result in more energy available; and therefore, in more energy allocation to defence and repairing compounds, since a large fraction of defence and repairing capacity is energy-dependent (Laureano et al. 2016).

For the study populations, all these variables (*GS*, S/R and SLA) are connected, as has been noticed previously (Chapter 2), where was found significant and negative

relationships between *PN* (and therefore *GS*) and S/R and SLA. These relationships have been considered as a compensation mechanism through which the populations with low photosynthetic efficiency would invest higher resources to the aerial fraction. The Cambo and Agadir populations showed the mean lowest normalized values of *GS*, as well as high normalized SLA and S/R values (Chapter 2); and consequently, they displayed the slightest general reduction in photochemical performance under ozone treatment.

On the other hand, the Alhamilla population had low normalized values of SLA and S/R, while the Leon population had intermediate values (Fig. S5 in Appendix S3). Although Alhamilla and León exhibited the highest normalized *GS* values, the former was the most photosynthetically affected by ozone, while latter was, in comparison, slightly affected (Fig. S2 in Appendix S3). These two populations differ in their native habitats. Thus, while the Alhamilla population is a milder one, the León population is native to a harsh environment characterized by chilling temperatures in winter, which could result in the selection of plants adapted to these stressful conditions. In fact, in a previous study carried out on León seedlings it was confirmed comparative high mesophyll endurance in this population when plants were fumigated with SO_2 (García *et al.*, 1998). These results highlight that the mechanisms underlying the plant responses to oxidant gases are quite complex as (besides to uptake) other factors are involved, e.g. the metabolic capacity to withstand oxidative stress (Lyons *et al.* 1997). In this sense, although we did not conducted any study of antioxidant systems, we have evidence of higher maintenance respiration rates (measured in mature leaves) in Cambo, Agadir and León populations compared with the rest (data not shown). These higher respiration rates could be closely related to higher defence endowments and repair mechanisms

(Laureano *et al.*, 2008, 2013 and 2016), altogether explaining the higher resistance of the above cited three populations.

5.3. Biogeographical patterns of ozone intraspecific sensitivity

According to expectations, our results showed intraspecific differences (physiological and morphological) in *Q. ilex* seedlings in response to ozone (Figs. 3 and S2-S4 in Appendix S3). Regarding the physiological variables, following the PCA results, Cambo and Agadir (edge populations, group I) were the most tolerant regarding the photochemical efficiency, although these edge populations were not exhibit the highest resistance in terms of biomass loss. For their part, the populations from the group II (Chaouen, Alhamilla, Font Roja and Salamanca) were quantitatively the most sensitive to ozone in terms of photochemical performance, as well as in seedling biomass loss. The remaining populations were included in the group III (León, Felix, Montblanc, Atzeneta, Beni Mellal and Pintado) and displayed intermediate sensitivity.

The populations inherently more tolerant to ozone stress (Cambo and Agadir) had convergent response to stress owing to exhibit the lowest variations caused by ozone in the most important surrogates of photochemical performance (e.g. $F_v/F_{m,}$ $\Phi_{PSII,}$ F_q', $q_{P,}$ *%D*, Fig. S4 in Appendix S3). Thereby, a range margin pattern was displayed on the response to ozone stress of *Q. ilex*, where populations native to peripheral locations maintained better photochemical performance under fumigation than populations native to core locations (Fig. 5). Our outcomes agree with the hypothesis formulated by Bassin *et al.* (2007) for grasslands, which postulated that communities from less productive habitats for the species ecological requirements (in our case, edge populations) could be less affected by ozone; while those from more favourable habitats could be more sensitive. In addition, it would be expected that more stressful habitats for the species

favours the selection of genotypes better adapted to high oxidative stress levels (e.g. cold and wet in the north of distribution range, and hot and dry in the south; Cambo and Agadir, respectively), i.e. the conservative syndrome so-called phenotypic canalization (Valladares et al. 2002b).

In this line, despite the harsh climatic conditions occurring at León and Felix locations (Table 1), both populations were grouped in the intermediate sensitivity group, instead of in the most tolerant group (Fig. 3). The response to treatment of the León seedlings seemed to be close to that showed by the two edge populations. However, the high stomatal aperture of León population likely has led to a high uptake of pollutant, resulting in a high decrease in F_m values compared with the most edge populations (Fig. S4 in Appendix S3). For its part, Felix showed the highest decrease in SLA (reduction in leaf surface combined with a slight increase leaf weight), which indicates the activation of a physical defence mechanism (Ribas et al. 2005b). Such behaviours make differences between both Felix and León populations and those remaining considered intermediate.

As tree biomass is the accumulated result of several processes occurring at cellular and physiological level throughout the life of the plant, biomass variations can be used as a valuable criterion to rank the intraspecific sensitivity to a pollutant (Alonso *et al.,* 2014). In this sense, the differential reduction of biomass fractions (roots, shoots or leaves) or total biomass observed among populations, suggests a gradual sensitivity in those in response to ozone stress. According with this biomass decrease, the PCA group II seem to be the most susceptible populations to ozone fumigation (Fig. S3 in Appendix S3). Moreover, these populations displayed the highest increase in *NPQ* values, which was significantly correlated to the highest decreases in seedlings total weight (Fig. 4). This higher sensitivity is not only related to high stomatal aperture

leading to higher ozone dose inside leaves, but also could be due to a putative less investment in defence, repair and compensation process in these populations.

5.4. Conclusions

The findings of this study show that *Q. ilex* seedlings have considerable tolerance to oxidative stress on the basis of the variety of regulatory mechanisms exhibited (stomatal closure, PSII down-regulation, heat dissipation, etc.). This result is expected in plants growing in the Mediterranean Basin (and, in general in plants native to Mediterranean type climate areas), which are adapted to several inducing oxidative stress factors (such as drought, high temperature, intense sun-light), since molecular basis of the response to these stressors may be convergent (cross-resistance) (García *et al.*, 1998; Paoletti 2006, Bussotti 2008). Furthermore, we found high intraspecific plasticity (in gas exchange, thermal dissipation, maximum PSII efficiency) and considerable differences concerning the physiological traits (and, to a lesser extent, the morphological ones) in reaction to ozone among the analyzed populations. These results are in the same line than those reported by Paludan-Müller *et al.* (1999) for 12 provenances of *Fagus sylvatica*. However, in contrast to these authors, which detected that the northern populations were more sensitive to ozone than the southern ones; we found that not only the southernmost population (Agadir), but also the northernmost one (Cambo) (namely the two edge populations), show the highest tolerance to ozone. Thus, the resistance to ozone stress displayed a range margin pattern compared with the latitudinal one showed by the cited authors. Our results suggest that defence endowments to stress related to range edge conditions (low maximum temperatures at northern edge, drought at southern edge) are capable of inducing resistance to other stressors, such as O_3 as well (Mittler, 2006). Bordering populations exhibited the highest tolerance by means of the lowest reduction of photochemical performance, obtained by their both lower gas exchange rates

(avoidance strategy) (Bussotti, 2008), and the putative constitutive ability to tolerate oxidative stress by an active antioxidant pool (Paoletti, 2006). By the opposite, core populations (especially, Chaouen, Salamanca, Font Roja, and Alhamilla) were the most affected, showing the highest loss in total seedling mass.

The results demonstrate the importance of the provenance level as a determinant of species stress resistance, and therefore, the potential outcome of new stressful scenarios, such as Climate Change, for the persistence of that particular species. Peripheral provenances, which seem to reveal more resistance to general stress, could become refugees for the species, and thus, a key alternative for future forests. The results got in this study on the response to ozone fumigation at intraspecific level, calls for additional studies on intraspecific Holm oak performance under other severe stress conditions (drought, high and low temperature, etc.), which this species likely may suffer in the near future.

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7. SUPPORTING INFORMATION

Appendix S1. Supplemental methods S1-S2

Methods S1 Chlorophyll *a* fluorescence measurement protocol

After 90 days of treatment, the same leaf per plant and per each population (from both Control and Treatment plants) was assessed by modulated chlorophyll *a* fluorescence inside growth chambers (n=15 leaves per population and per treatment). For light measurements we adapted selected leaves at 450 µmol $m^{-2}s^{-1}$ for one hour to ensure all samples were measured at the same conditions. After that, fluorescence emission was recorded at measured leaf during actinic illumination (F_s), followed by a saturating pulse that provides the maximum fluorescence during actinic illumination (F_m'). F_o' was calculated as Oxborough & Baker 1997. Subsequently, measured leaf was dark adapted for 30 minutes to *Qa* becomes maximally oxidized and the basal fluorescence (F_o) upon excitation of measured leaf with a weak non-actinic beam was obtained. The maximum fluorescence (F_m) was obtained following a pulse of saturating light to maximally reduce *Qa*. Variable fluorescence (F_v) was determined as the difference between F_m and F_o, and the maximum quantum yield of *Qa* reduction was estimated as the ratio F_v/F_m.

The actual quantum yield of PSII photochemistry, Φ_{PSII}, closely associated with the quantum yield of non-cyclic electron transport, was estimated from $(F_m' - F_s)/ F_m'$ (Genty *et al*.1989) and the intrinsic PSII efficiency (F_v'/F_m') was calculated as $(F_m' - F_o')/ F_m'$. Photochemical quenching (F_q'), related to the amount of electrons flowing through the electron transport chain, was calculated as $F_m' - F_s$ (Baker, 2008). Non-photochemical quenching (*NPQ*) is related to dissipation of absorbed light energy, and was determined according to the equation $NPQ = Fm / F_m' - 1$ (Bilger and Björkman 1990). The coefficient of photochemical quenching (q_P), related to the fraction of open PSII reaction centres, was estimated as $(F_m' - Fs)/(F_m' - F_o')$ (Schreiber *et al*. 1986). The ratio $(1 - q_P)/NPQ$ was used as an estimation of photon excess and, therefore, as the susceptibility of PSII to irradiance (Park *et al*. 1995). The fractions of the light absorbed dissipated as heat in the PSII antennae (%*D*) was calculated as $1 - (F_v'/F_m') \times 100$ and those

utilized in PSII photochemistry (%*P*) were estimated as $(F_v'/F_m')q_P \times 100$. The fraction of excitation energy not dissipated termally in the antennae that cannot be used in photochemistry pathway (%*X*) was estimated as (F_v'/F_m') x $(1 - q_P) \times 100$ (Demmig-Adams, 1996).

Methods S2 Data normalization

In order to avoid finding differences among populations caused by different growth conditions between years (2010 and 2013), we performed the data normalization of the relative value expressed as treated/control ratio for each population per year by using the following formula:

$$X' = \frac{X - Xm}{Xsd}$$

where *X*' is the normalized value of a given variable, *X* represents the original value of the response to ozone, X_m is the average value for such variable per year, and X_{SD} is the standard deviation.

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Appendix S2. Supplemental tables S1-S4

Table S1. Average ozone effects relative Control to Treatment (%) in the main variables studied for the twelve *Q. ilex* populations considered. Numbers in bold denote significant differences ($P < 0.05$) between C and T per population (U-Mann Withney Test). Asteriks indicate significant effects of ozone, population and their interaction: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, $P < 0.1^{m.s.}$.

	2010						Significant effects			2013						Significant effects		
	Felix	**Alhamilla**	**Font Roja**	**Atzeneta**	**Montblanc**	**Cambo**	**O_3**	**Pop**	**O_3 x Pop**	**Agadir**	**Beni Mellal**	**Chaouen**	**Sevilla**	**Salamanca**	**León**	**O_3**	**Pop**	**O_3 x Pop**
PN (µmol CO_2 $cm^2 g^{-1}$)	**-20.2** ± 4.8	**-36.7** ± 4.1	**-26.2** ± 4.4	**-35.6** ± 3.3	**-18.0** ± 3.6	-11.7 ± 3.3	***	**	***	**-50.4** ± 5.1	**-42.5** ± 4.9	**-45.9** ± 6.1	**-58.2** ± 4.9	**-56.0** ± 5.5	**-49.2** ± 4.1	***	**	n.s.
Gs (mmol H_2O $cm^2 g^{-1}$)	**-31.2** ± 6.7	**-30.1** ± 5.0	**-18.1** ± 4.7	**-42.7** ± 4.9	**-15.0** ± 4.9	-9.9 ± 5.2	***	n.s.	**	**-35.3** ± 7.1	-15.5 ± 7.6	-16.7 ± 8.0	-11.5 ± 10.1	**-28.5** ± 7.2	-11.2 ± 6.7	***	***	n.s.
Ci (µmol mol^{-1})	1.5 ± 1.9	**7.8** ± 1.7	**16.0** ± 2.1	2.9 ± 2.1	**3.6** ± 1.7	**9.6** ± 2.0	***	*	***	**57.0** ± 15.3	**45.5** ± 4.2	**74.0** ± 13.3	**70.4** ± 5.3	**98.3** ± 9.4	**47.6** ± 5.8	***	***	*
WUE (µmol $mmol^{-1}$)	**20.3** ± 7.1	-8.4 ± 5.5	-10.7 ± 5.3	**19.8** ± 8.0	-2.3 ± 4.5	-0.8 ± 5.8	n.s.	n.s.	**	**-22.1** ± 6.6	**-36.2** ± 4.3	**-35.3** ± 6.4	**-58.6** ± 4.0	**-42.1** ± 4.5	**-41.6** ± 6.0	***	***	n.s.
Fo	-4.1 ± 1.9	6.7 ± 3.7	2.3 ± 2.1	2.3 ± 2.8	-1.1 ± 2.3	-1.4 ± 2.1	n.s.	*	n.s.	1.2 ± 3.4	3.5 ± 2.9	-1.9 ± 5.8	-2.2 ± 5.4	2.1 ± 4.7	-6.6 ± 4.8	n.s.	n.s.	n.s.
Fm	-5.6 ± 2.1	-1.8 ± 2.4	1.5 ± 1.5	-1.1 ± 1.9	**-6.1** ± 1.8	-0.7 ± 2.0	*	n.s.	n.s.	**-12.7** ± 2.6	**-18.7** ± 3.4	**-21.2** ± 4.4	**-30.4** ± 4.4	**-20.9** ± 3.6	**-27.1** ± 4.0	***	n.s.	m.s.
Fv	-5.9 ± 2.5	-3.8 ± 2.4	1.3 ± 1.7	-1.9 ± 2.0	**-7.3** ± 1.9	-0.6 ± 2.3	**	n.s.	n.s.	**-15.9** ± 2.8	**-23.7** ± 3.9	**-25.7** ± 4.3	**-36.8** ± 4.4	**-26.2** ± 3.8	**-31.6** ± 4.1	***	n.s.	m.s.
Fv/Fm	-0.5 ± 0.6	-1.9 ± 0.5	-0.2 ± 0.5	-0.8 ± 0.5	**-1.2** ± 0.4	0.1 ± 0.5	**	*	n.s.	**-3.7** ± 0.8	**-6.8** ± 1.2	**-5.8** ± 0.8	**-9.9** ± 1.1	**-7.0** ± 1.2	**-6.5** ± 0.8	***	n.s.	n.s.
ΦPSII	**-12.2** ± 3.2	**-11.2** ± 3.8	**-15.3** ± 3.9	**-15.3** ± 3.6	-8.5 ± 4.4	-1.8 ± 4.9	***	***	n.s.	**-29.2** ± 4.3	**-43.1** ± 4.4	**-42.6** ± 2.9	**-47.2** ± 3.3	**-40.8** ± 3.7	**-34.3** ± 3.8	***	n.s.	n.s.
Fs	**-12.5** ± 6.3	**-22.8** ± 5.1	**-20.1** ± 5.3	**-10.1** ± 4.9	-9.5 ± 4.6	-11.2 ± 6.9	***	**	n.s.	**-31.4** ± 3.3	**-37.6** ± 2.9	**-43.3** ± 4.3	**-40.1** ± 3.5	**-48.0** ± 2.8	**-42.8** ± 3.1	***	**	*
Fm'	**-17.8** ± 5.8	**-27.0** ± 4.8	**-24.8** ± 5.4	**-16.8** ± 4.7	-12.0 ± 5.4	-12.0 ± 6.5	***	*	n.s.	**-38.7** ± 3.4	**-48.2** ± 2.7	**-53.5** ± 3.4	**-50.5** ± 3.1	**-56.2** ± 2.7	**-50.8** ± 2.7	***	**	*
Fq'	**-27.7** ± 5.5	**-36.6** ± 5.0	**-35.1** ± 6.6	**-28.9** ± 5.9	-17.6 ± 8.2	-14.3 ± 7.6	***	**	n.s.	**-55.5** ± 4.8	**-69.4** ± 3.4	**-73.5** ± 2.2	**-72.9** ± 2.7	**-72.9** ± 3.0	**-67.3** ± 2.9	***	**	**
qP	-6.8 ± 3.5	0.8 ± 4.1	-6.5 ± 4.1	**-9.3** ± 3.4	-4.4 ± 3.7	2.9 ± 5.0	*	***	n.s.	**-14.1** ± 3.7	**-24.8** ± 4.6	**-22.0** ± 4.2	**-28.7** ± 3.8	**-17.3** ± 3.5	**-17.3** ± 4.4	***	n.s.	n.s.
NPQ	65.5 ± 28.2	**128.7** ± 26.4	**187.6** ± 47.2	**53.7** ± 15.9	23.0 ± 15.2	48.1 ± 26.6	***	**	m.s.	**78.0** ± 13.5	**98.4** ± 8.6	**126.9** ± 15.0	**62.3** ± 10.3	**194.0** ± 16.7	**90.7** ± 15.3	***	**	*
(1-qP)/NPQ	-11.2 ± 16.5	**-57.6** ± 6.1	**-61.7** ± 6.4	-27.6 ± 9.5	-5.3 ± 15.0	-20.5 ± 16.3	***	***	n.s.	**-43.1** ± 4.3	**-44.3** ± 2.7	**-67.2** ± 2.7	**-39.2** ± 5.0	**-67.7** ± 1.7	**-51.5** ± 6.9	***	**	*
%D	16.0 ± 6.8	**35.4** ± 6.0	**31.0** ± 8.3	**17.6** ± 5.7	11.1 ± 5.1	11.2 ± 7.5	***	***	n.s.	**37.4** ± 5.3	**55.9** ± 4.9	**59.7** ± 4.9	**56.2** ± 4.7	**74.6** ± 5.8	**53.3** ± 5.1	***	***	n.s.
%X	0.7 ± 4.6	**-11.9** ± 3.4	-4.6 ± 4.1	1.6 ± 3.9	-1.6 ± 3.0	-5.3 ± 4.4	n.s.	***	n.s.	-9.5 ± 3.3	-9.0 ± 3.7	-7.7 ± 4.7	-8.2 ± 3.6	**-19.8** ± 2.3	-9.6 ± 4.0	***	n.s.	n.s.
Above-ground biomass (g)	-15.6 ± 8.5	**-18.2** ± 8.6	-11.5 ± 9.2	-5.8 ± 9.3	-8.3 ± 7.7	-5.1 ± 6.0	m.s.	***	n.s.	**-22.7** ± 2.1	-8.3 ± 1.9	**-16.5** ± 2.3	0.0 ± 2.9	**-33.9** ± 1.6	**-24.8** ± 1.9	***	***	n.s.
Roots (g)	1.0 ± 9.3	-16.1 ± 6.7	-14.8 ± 7.4	-3.5 ± 8.3	0.9 ± 4.6	-11.1 ± 5.8	n.s.	***	n.s.	**-42.3** ± 1.1	**-27.2** ± 1.6	**-47.4** ± 1.2	-33.2 ± 2.2	**-52.8** ± 1.1	**-40.5** ± 1.4	***	***	m.s.
Total mass (g)	-10.0 ± 7.9	**-17.3** ± 6.7	-12.6 ± 7.3	-4.9 ± 7.1	-5.3 ± 6.2	-6.7 ± 4.6	*	***	n.s.	**-30.6** ± 1.6	-17.1 ± 1.6	**-30.7** ± 1.7	-16.6 ± 2.5	**-43.0** ± 1.3	**-31.8** ± 1.6	***	***	n.s.
LS	-9.6 ± 4.5	-6.9 ± 4.4	4.0 ± 9.4	10.8 ± 6.8	3.9 ± 7.6	5.5 ± 6.0	n.s.	***	n.s.	**-16.7** ± 8.9	7.8 ± 8.2	-6.9 ± 5.7	-3.1 ± 7.7	**-22.3** ± 6.2	-6.4 ± 6.4	*	***	n.s.
LW	4.1 ± 5.9	-4.9 ± 5.0	-2.1 ± 8.1	6.8 ± 6.9	2.6 ± 5.3	6.9 ± 6.2	n.s.	***	n.s.	-13.6 ± 9.5	12.9 ± 9.6	4.8 ± 7.2	5.3 ± 8.9	-14.1 ± 6.6	-3.5 ± 6.7	n.s.	***	n.s.

	2010						Significant effects			2013						Significant effects		
	Felix	**Alhamilla**	**Font Roja**	**Atzeneta**	**Montblanc**	**Cambo**	**O_3**	**Pop**	**O_3 x Pop**	**Agadir**	**Beni Mellal**	**Chaouen**	**Sevilla**	**Salamanca**	**Leon**	**O_3**	**Pop**	**O_3 x Pop**
SLA (cm^2g^{-1})	**-12.9** ± 2.0	-1.4 ± 2.5	6.0 ± 3.3	3.8 ± 2.1	-1.7 ± 2.9	-2.5 ± 1.4	n.s.	***	***	-2.5 ± 3.2	-3.0 ± 2.6	-1.6 ± 2.7	**-7.2** ± 2.5	**-8.5** ± 2.3	-2.5 ± 2.5	***	***	n.s.
LA (cm^2)	-22.5 ± 8.1	**-13.9** ± 8.1	-1.9 ± 11.8	-5.5 ± 10.5	-2.1 ± 5.2	-3.3 ± 6.4	n.s.	m.s.	n.s.	-18.3 ± 10.7	-3.9 ± 9.7	-16.0 ± 8.7	9.2 ± 11.6	-26.6 ± 6.8	-13.0 ± 9.2	*** *	** **	n.s. n.s.
S/R (gg^{-1})	-14.6 ± 7.0	-4.5 ± 8.7	11.5 ± 11.1	4.2 ± 11.8	-13.4 ± 5.8	12.7 ± 12.0	n.s.	***	n.s.	**29.0** ± 2.6	28.6 ± 2.6	**57.7** ± 3.5	**48.4** ± 2.6	**43.8** ± 1.9	13.4 ± 1.9			
LAR (cm^2g^{-1})	**-15.7** ± 3.3	0.3 ± 5.8	9.7 ± 5.7	1.0 ± 6.9	-1.0 ± 3.5	1.7 ± 3.4	n.s.	***	n.s.	15.6 ± 6.1	14.0 ± 7.1	**33.4** ± 6.2	23.4 ± 6.1	**28.9** ± 6.7	7.9 ± 5.7	***	***	n.s.
LWR (gg^{-1})	-4.2 ± 3.8	-0.8 ± 5.7	3.0 ± 4.0	-3.9 ± 6.1	-2.0 ± 3.0	3.6 ± 2.6	n.s.	***	n.s.	**18.0** ± 4.5	16.3 ± 6.0	**35.7** ± 5.8	**31.8** ± 5.1	**30.8** ± 6.5	6.9 ± 4.7	***	**	n.s.
n	15	15	16	14	15	15				20	20	17	20	19	20			

Table S2. Average ozone effects relative Control to Treatment (%) in the main variables studied in *Q. ilex* for each year of study. Asteriks indicate significant effects of ozone, population and their interaction: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, $P < 0.1^{m.s.}$.

		Significant effects				Significant effects		
%	**2010**	**O_3**	**Pop**	**O_3 x Pop**	**2013**	**O_3**	**Pop**	**O_3 x Pop**
PN (µmol CO_2 cm^2g^{-1})	-24.7 ± 1.8	***	**	***	-49.8 ± 2.1	***	**	n.s.
Gs (mmol H_2O cm^2g^{-1})	-24.5 ± 2.4	***	n.s.	**	-19.2 ± 3.2	***	***	n.s.
Ci (µmol mol^{-1})	6.9 ± 0.9	***	*	***	63.8 ± 4.1	***	***	*
WUE (µmol $mmol^{-1}$)	3.0 ± 2.8	n.s.	n.s.	**	-39.0 ± 2.3	***	***	n.s.
Fo	0.8 ± 1.1	n.s.	*	n.s.	-0.6 ± 1.9	n.s.	n.s.	n.s.
Fm	-2.3 ± 0.8	*	n.s.	n.s.	-21.8 ± 1.6	***	n.s.	m.s.
Fv	-3.0 ± 0.9	**	n.s.	n.s.	-26.6 ± 1.7	***	n.s.	m.s.
Fv/Fm	-0.8 ± 0.2	**	*	n.s.	-6.6 ± 0.4	***	n.s.	n.s.
ΦPSII	-10.7 ± 1.7	***	***	n.s.	-39.3 ± 17.4	***	n.s.	n.s.
Fs	-14.4 ± 2.3	***	**	n.s.	-40.5 ± 1.4	***	**	*
Fm'	-18.4 ± 2.2	***	*	n.s.	-49.6 ± 1.3	***	**	*
Fq'	-26.7 ± 2.8	***	**	n.s.	-68.2 ± 15.4	***	**	**
qP	-3.8 ± 1.7	*	***	n.s.	-20.7 ± 18.1	***	n.s.	n.s.
NPQ	84.8 ± 12.9	***	**	m.s.	104.7 ± 67.7	***	**	*
(1-qP)/NPQ	-30.3 ± 5.5	***	***	n.s.	-52.0 ± 2.0	***	**	*
%D	20.4 ± 2.8	***	***	n.s.	56.2 ± 2.3	***	***	n.s.
%X	-3.6 ± 1.6	n.s.	***	n.s.	-10.8 ± 1.5	***	n.s.	n.s.
Above-ground biomass (g)	-12.2 ± 3.6	m.s.	***	n.s.	-17.7 ± 3.9	***	***	n.s.
Roots (g)	-10.7 ± 3.3	n.s.	***	n.s.	-39.9 ± 2.8	***	***	m.s.
Total mass (g)	-9.5 ± 2.7	*	***	n.s.	-28.2 ± 3.2	***	***	n.s.
LS (cm^2)	-0.1 ± 2.4	n.s.	***	n.s.	-7.8 ± 3.0	*	***	n.s.
LW (g)	2.3 ± 2.5	n.s.	***	n.s.	-1.3 ± 3.4	n.s.	***	n.s.
SLA (cm^2g^{-1})	-1.5 ± 1.2	n.s.	***	***	-4.2 ± 1.1	***	***	n.s.
LA (cm^2)	-8.0 ± 3.5	n.s.	m.s.	n.s.	-11.5 ± 4.0	*	**	n.s.
S/R (gg^{-1})	0.4 ± 4.1	n.s.	***	n.s.	36.0 ± 4.6	***	**	n.s.
LAR (cm^2g^{-1})	-0.5 ± 2.1	n.s.	***	n.s.	20.3 ± 2.7	***	***	n.s.
LWR (gg^{-1})	-0.6 ± 1.8	n.s.	***	n.s.	22.8 ± 2.4	***	**	n.s.
n	90				116			

Table S3. Mean values (± SE) of Control and Treatment plants in *Q. ilex* populations

	Agadir		Beni Mellal		Chaouen		Sevilla		Salamanca		Leon	
	C	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**
PN (µmol CO_2 cm^2g^{-1})	**11.00** ± 0.73	**5.38** ± 0.55	**12.00** ± 0.67	**6.90** ± 0.59	**11.12** ± 0.99	**6.02** ± 0.67	**12.74** ± 0.66	**5.35** ± 0.57	**13.89** ± 0.51	**6.12** ± 0.76	**13.60** ± 0.45	**6.90** ± 0.56
Gs (mmol H_2O cm^2g^{-1})	**59.6** ± 4.1	**38.2** ± 4.2	**95.9** ± 9.2	**81.0** ± 7.3	**62.5** ± 6.1	**52.0** ± 5.0	**102.3** ± 10.9	**87.5** ± 9.6	**81.3** ± 5.4	**58.2** ± 5.9	**110.2** ± 7.8	**97.8** ± 7.4
Ci (µmol mol^{-1})	**125** ± 16	**201** ± 20	**191** ± 15	**278** ± 8	**152** ± 22	**265** ± 20	**187** ± 17	**316** ± 9	**139** ± 12	**275** ± 13	**191** ± 13	**282** ± 11
WUE (µmol $mmol^{-1}$)	**0.190** ± 0.011	**0.147** ± 0.012	**0.141** ± 0.012	**0.090** ± 0.006	**0.183** ± 0.012	**0.119** ± 0.012	**0.144** ± 0.012	**0.063** ± 0.006	**0.183** ± 0.008	**0.106** ± 0.008	**0.135** ± 0.010	**0.079** ± 0.008
Fo	**168** ± 3	**170** ± 6	**167** ± 3	**173** ± 5	**173** ± 3	**170** ± 10	**170** ± 4	**166** ± 9	**172** ± 3	**176** ± 8	**175** ± 3	**163** ± 8
Fm	**903** ± 18	**786** ± 24	**912** ± 11	**742** ± 31	**925** ± 13	**729** ± 40	**918** ± 13	**639** ± 41	**921** ± 14	**728** ± 33	**964** ± 13	**703** ± 38
Fv	**735** ± 17	**616** ± 21	**745** ± 9	**569** ± 29	**752** ± 10	**559** ± 32	**748** ± 12	**473** ± 33	**749** ± 13	**553** ± 28	**790** ± 12	**540** ± 32
Fv/Fm	**0.813** ± 0.004	**0.782** ± 0.006	**0.817** ± 0.003	**0.761** ± 0.010	**0.813** ± 0.002	**0.766** ± 0.006	**0.815** ± 0.004	**0.734** ± 0.009	**0.813** ± 0.003	**0.756** ± 0.010	**0.818** ± 0.002	**0.765** ± 0.007
ΦPSII	**0.300** ± 0.009	**0.214** ± 0.013	**0.333** ± 0.014	**0.189** ± 0.015	**0.335** ± 0.012	**0.192** ± 0.009	**0.316** ± 0.014	**0.167** ± 0.010	**0.331** ± 0.014	**0.196** ± 0.012	**0.329** ± 0.012	**0.216** ± 0.012
Fv'/Fm'	**0.673** ± 0.014	**0.548** ± 0.018	**0.686** ± 0.014	**0.511** ± 0.015	**0.695** ± 0.017	**0.513** ± 0.014	**0.681** ± 0.015	**0.502** ± 0.015	**0.717** ± 0.012	**0.505** ± 0.016	**0.718** ± 0.015	**0.567** ± 0.014
Fs	**308** ± 18	**210** ± 10	**312** ± 21	**195** ± 9	**341** ± 25	**194** ± 14	**318** ± 20	**191** ± 11	**370** ± 20	**192** ± 11	**382** ± 23	**218** ± 11
Fm'	**442** ± 26	**270** ± 15	**468** ± 30	**243** ± 12	**515** ± 36	**240** ± 17	**467** ± 28	**231** ± 15	**552** ± 26	**242** ± 15	**568** ± 31	**279** ± 15
Fo'	**138** ± 3	**118** ± 4	**140** ± 3	**116** ± 4	**147** ± 3	**114** ± 7	**142** ± 4	**112** ± 6	**151** ± 3	**116** ± 5	**153** ± 3	**119** ± 5
Fq'	**133.7** ± 9.5	**59.7** ± 6.7	**156.0** ± 11.4	**47.8** ± 5.3	**173.6** ± 14.3	**46.0** ± 3.7	**148.5** ± 11.5	**40.3** ± 4.0	**182.6** ± 11.3	**49.5** ± 5.5	**186.2** ± 10.9	**60.9** ± 5.2
qP	**0.447** ± 0.014	**0.388** ± 0.017	**0.487** ± 0.022	**0.366** ± 0.023	**0.483** ± 0.017	**0.377** ± 0.019	**0.466** ± 0.020	**0.332** ± 0.018	**0.462** ± 0.020	**0.382** ± 0.016	**0.462** ± 0.019	**0.383** ± 0.020
NPQ	**1.151** ± 0.107	**2.043** ± 0.159	**1.067** ± 0.109	**2.118** ± 0.092	**0.953** ± 0.148	**2.163** ± 0.139	**1.118** ± 0.141	**1.815** ± 0.115	**0.717** ± 0.092	**2.110** ± 0.120	**0.817** ± 0.130	**1.557** ± 0.121
(1-qP)/NPQ	**0.592** ± 0.082	**0.332** ± 0.026	**0.550** ± 0.084	**0.307** ± 0.015	**0.882** ± 0.178	**0.289** ± 0.023	**0.666** ± 0.097	**0.405** ± 0.033	**0.956** ± 0.101	**0.309** ± 0.016	**0.989** ± 0.145	**0.479** ± 0.066
%D	**32.7** ± 1.4	**45.2** ± 1.8	**31.4** ± 1.4	**48.9** ± 1.5	**30.5** ± 1.7	**48.7** ± 1.4	**31.9** ± 1.5	**49.8** ± 1.5	**28.3** ± 1.2	**49.5** ± 1.6	**28.2** ± 1.5	**43.3** ± 1.4
%X	**37.3** ± 1.4	**33.4** ± 1.2	**35.4** ± 1.8	**32.2** ± 1.3	**34.8** ± 1.3	**32.1** ± 1.6	**36.5** ± 1.7	**33.5** ± 1.3	**38.6** ± 1.7	**31.0** ± 0.9	**38.8** ± 1.9	**35.1** ± 1.5
Leaves (g)	**2.18** ± 0.25	**1.81** ± 0.25	**2.37** ± 0.22	**2.35** ± 0.24	**2.34** ± 0.21	**2.21** ± 0.24	**1.78** ± 0.25	**1.95** ± 0.23	**2.48** ± 0.29	**1.88** ± 0.20	**1.69** ± 0.14	**1.30** ± 0.16
Stem (g)	**1.31** ± 0.18	**0.79** ± 0.09	**1.52** ± 0.16	**1.22** ± 0.11	**1.63** ± 0.15	**1.12** ± 0.16	**1.03** ± 0.17	**0.84** ± 0.13	**1.72** ± 0.18	**0.86** ± 0.10	**0.84** ± 0.08	**0.57** ± 0.06
Above-ground biomass (g)	**3.42** ± 0.38	**2.59** ± 0.33	**3.90** ± 0.37	**3.58** ± 0.33	**3.99** ± 0.33	**3.33** ± 0.38	**2.81** ± 0.41	**2.82** ± 0.36	**4.20** ± 0.46	**2.78** ± 0.31	**2.51** ± 0.19	**1.89** ± 0.22
Roots (g)	**2.33** ± 0.21	**1.31** ± 0.12	**3.34** ± 0.31	**2.43** ± 0.24	**3.40** ± 0.23	**1.79** ± 0.17	**2.83** ± 0.36	**1.89** ± 0.28	**3.91** ± 0.26	**1.84** ± 0.19	**2.04** ± 0.23	**1.21** ± 0.12
Total mass (g)	**5.75** ± 0.53	**3.90** ± 0.42	**7.25** ± 0.65	**6.01** ± 0.51	**7.39** ± 0.49	**5.12** ± 0.51	**5.65** ± 0.73	**4.71** ± 0.62	**8.11** ± 0.63	**4.62** ± 0.47	**4.55** ± 0.35	**3.10** ± 0.32
LS (cm^2)	**7.99** ± 0.47	**6.62** ± 0.74	**6.29** ± 0.47	**6.77** ± 0.51	**6.87** ± 0.33	**6.40** ± 0.39	**5.04** ± 0.36	**4.88** ± 0.38	**5.97** ± 0.42	**4.64** ± 0.35	**4.92** ± 0.27	**4.72** ± 0.28
LW (g)	**0.117** ± 0.008	**0.100** ± 0.012	**0.097** ± 0.008	**0.109** ± 0.009	**0.093** ± 0.006	**0.097** ± 0.007	**0.079** ± 0.006	**0.083** ± 0.007	**0.097** ± 0.007	**0.083** ± 0.006	**0.075** ± 0.004	**0.075** ± 0.004
SLA (cm^2g^{-1})	**69.1** ± 1.6	**67.8** ± 2.4	**65.7** ± 1.4	**63.7** ± 1.7	**67.7** ± 4.5	**66.7** ± 1.8	**64.1** ± 1.0	**59.5** ± 1.5	**61.1** ± 1.2	**55.8** ± 1.4	**65.8** ± 1.1	**63.2** ± 1.3
LA (cm^2)	**150** ± 17	**121** ± 17	**155** ± 15	**149** ± 15	**172** ± 13	**144** ± 15	**112** ± 15	**116** ± 13	**156** ± 18	**110** ± 11	**111** ± 9	**93** ± 9
S/R (gg^{-1})	**1.50** ± 0.11	**1.96** ± 0.19	**1.22** ± 0.09	**1.57** ± 0.14	**1.22** ± 0.10	**1.92** ± 0.18	**1.10** ± 0.09	**1.64** ± 0.13	**1.08** ± 0.10	**1.56** ± 0.09	**1.39** ± 0.12	**1.57** ± 0.12
LAR (cm^2g^{-1})	**25.8** ± 1.1	**30.2** ± 1.7	**21.7** ± 1.0	**24.7** ± 1.5	**21.3** ± 1.6	**28.4** ± 1.3	**21.0** ± 1.4	**23.3** ± 2.1	**17.0** ± 1.5	**22.5** ± 1.0	**25.0** ± 1.4	**26.4** ± 1.3
LWR (gg^{-1})	**0.374** ± 0.016	**0.445** ± 0.018	**0.331** ± 0.016	**0.386** ± 0.020	**0.316** ± 0.016	**0.429** ± 0.018	**0.328** ± 0.021	**0.433** ± 0.017	**0.294** ± 0.018	**0.409** ± 0.017	**0.382** ± 0.022	**0.407** ± 0.019
n	19	18	19	20	17	17	19	20	19	20	19	20

To be continue

Table S3. *Continue*

	Felix		Alhamilla		Font Roja		Atzeneta		Montblanc		Cambó	
	C	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**
PN (µmol CO_2 cm^2g^{-1})	**15.22** ± 0.47	**12.14** ± 0.74	**16.74** ± 0.40	**10.60** ± 0.68	**16.26** ± 0.39	**12.01** ± 0.71	**15.97** ± 0.64	**10.29** ± 0.53	**14.28** ± 0.31	**11.71** ± 0.52	**13.23** ± 0.72	**11.68** ± 0.43
Gs (mmol H_2O cm^2g^{-1})	**316** ± 17	**217** ± 21	**312** ± 16	**219** ± 15	**284** ± 14	**232** ± 13	**313** ± 13	**180** ± 15	**276** ± 10	**234** ± 14	**246** ± 14	**222** ± 13
Ci (µmol mol^{-1})	**273** ± 5	**277** ± 5	**273** ± 3	**295** ± 5	**254** ± 4	**294** ± 5	**269** ± 3	**277** ± 6	**281** ± 3	**291** ± 5	**264** ± 6	**290** ± 5
WUE (µmol $mmol^{-1}$)	**0.050** ± 0.003	**0.006** ± 0.004	**0.055** ± 0.002	**0.051** ± 0.003	**0.059** ± 0.003	**0.053** ± 0.003	**0.051** ± 0.001	**0.061** ± 0.004	**0.052** ± 0.001	**0.051** ± 0.002	**0.055** ± 0.003	**0.055** ± 0.003
Fo	**162** ± 5	**155** ± 3	**156** ± 3	**167** ± 6	**148** ± 5	**152** ± 3	**155** ± 3	**159** ± 4	**166** ± 4	**164** ± 4	**158** ± 3	**156** ± 3
Fm	**871** ± 16	**822** ± 19	**841** ± 16	**826** ± 20	**818** ± 17	**830** ± 12	**836** ± 15	**819** ± 15	**879** ± 15	**825** ± 16	**850** ± 10	**844** ± 17
Fv	**709** ± 14	**667** ± 18	**685** ± 14	**659** ± 16	**670** ± 13	**678** ± 11	**682** ± 13	**660** ± 13	**713** ± 14	**661** ± 14	**692** ± 9	**688** ± 16
Fv/Fm	**0.814** ± 0.005	**0.810** ± 0.005	**0.814** ± 0.003	**0.798** ± 0.004	**0.819** ± 0.003	**0.817** ± 0.004	**0.815** ± 0.003	**0.806** ± 0.004	**0.811** ± 0.004	**0.801** ± 0.004	**0.814** ± 0.004	**0.814** ± 0.004
ΦPSII	**0.352** ± 0.007	**0.309** ± 0.011	**0.306** ± 0.012	**0.267** ± 0.010	**0.319** ± 0.012	**0.271** ± 0.012	**0.358** ± 0.013	**0.297** ± 0.012	**0.303** ± 0.014	**0.277** ± 0.013	**0.268** ± 0.015	**0.263** ± 0.013
Fv'/Fm'	**0.750** ± 0.009	**0.710** ± 0.017	**0.753** ± 0.009	**0.668** ± 0.016	**0.775** ± 0.007	**0.705** ± 0.019	**0.730** ± 0.009	**0.682** ± 0.014	**0.710** ± 0.009	**0.678** ± 0.015	**0.737** ± 0.012	**0.707** ± 0.020
Fs	**390** ± 13	**341** ± 25	**411** ± 15	**318** ± 21	**427** ± 15	**342** ± 23	**334** ± 14	**305** ± 16	**351** ± 12	**318** ± 16	**408** ± 20	**363** ± 28
Fm'	**602** ± 19	**495** ± 35	**594** ± 20	**434** ± 29	**627** ± 15	**471** ± 34	**520** ± 19	**436** ± 23	**505** ± 16	**445** ± 27	**561** ± 28	**493** ± 36
Fo'	**149** ± 4	**136** ± 3	**144** ± 2	**139** ± 5	**140** ± 4	**130** ± 3	**139** ± 3	**134** ± 3	**145** ± 3	**138** ± 3	**143** ± 3	**135** ± 3
Fq'	**212** ± 8	**153** ± 12	**182** ± 10	**116** ± 9	**199** ± 28	**129** ± 13	**186** ± 10	**131** ± 10	**154** ± 9	**127** ± 13	**152** ± 12	**130** ± 12
qP	**0.470** ± 0.010	**0.438** ± 0.016	**0.406** ± 0.015	**0.402** ± 0.016	**0.412** ± 0.016	**0.385** ± 0.017	**0.490** ± 0.017	**0.436** ± 0.016	**0.426** ± 0.018	**0.408** ± 0.016	**0.363** ± 0.020	**0.374** ± 0.018
NPQ	**0.467** ± 0.056	**0.773** ± 0.132	**0.447** ± 0.069	**1.007** ± 0.125	**0.312** ± 0.033	**0.898** ± 0.147	**0.640** ± 0.069	**0.947** ± 0.379	**0.769** ± 0.069	**0.946** ± 0.0.117	**0.579** ± 0.089	**0.857** ± 0.595
(1-qP)/NPQ	**1.218** ± 0.112	**1.081** ± 0.200	**1.718** ± 0.305	**0.873** ± 0.202	**2.292** ± 0.328	**1.128** ± 0.285	**0.942** ± 0.418	**0.716** ± 0.088	**0.851**± 0.093	**0.806** ± 0.127	**1.519** ± 0.244	**1.207** ± 0.248
%D	**25.0** ± 0.9	**29.0** ± 1.7	**24.7** ± 0.9	**33.2** ± 1.6	**22.5** ± 0.7	**29.5** ± 1.9	**27.0** ± 0.9	**31.8** ± 1.4	**29.0** ± 0.9	**32.2** ± 1.5	**26.3** ± 1.2	**29.3** ± 2.0
%X	**39.8** ± 1.0	**40.1** ± 1.9	**44.7** ± 1.2	**40.0** ± 1.6	**45.6** ± 1.3	**43.5** ± 1.9	**37.2** ± 1.3	**38.5** ± 1.4	**40.7** ± 1.3	**40.1** ± 1.2	46.9± 1.6	**44.4** ± 2.1
Leaves (g)	**2.92** ± 0.33	**2.45** ± 0.23	**2.84** ± 0.13	**2.39** ± 0.26	**2.27** ± 0.33	**2.01** ± 0.22	**3.46** ± 0.40	**3.09** ± 0.32	**3.42** ± 0.23	**3.25** ± 0.26	**4.44** ± 0.25	**4.31** ± 0.25
Stem (g)	**1.70** ± 0.20	**1.45** ± 0.16	**1.36** ± 0.07	**1.15** ± 0.12	**1.30** ± 0.22	**1.12** ± 0.13	**2.24** ± 0.27	**2.13** ± 0.22	**2.13** ± 0.18	**1.86** ± 0.18	**2.85** ± 0.19	**2.51** ± 0.20
Above-ground biomass (g)	**4.63** ± 0.50	**3.91** ± 0.38	**4.21** ± 0.18	**3.44** ± 0.36	**3.58** ± 0.54	**3.16** ± 0.34	**5.57** ± 0.62	**5.25** ± 0.52	**5.58** ± 0.38	**5.12** ± 0.43	**7.30** ± 0.41	**6.92** ± 0.43
Roots (g)	**2.38** ± 0.19	**2.41** ± 0.21	**3.33** ± 0.23	**2.79** ± 0.22	**1.92** ± 0.17	**1.64** ± 0.15	**3.27** ± 0.22	**3.15** ± 0.27	**2.72** ± 0.20	**2.75** ± 0.13	**2.64** ± 0.17	**2.35** ± 0.15
LS (cm^2)	**5.15** ± 0.45	**4.66** ± 0.23	**6.32** ± 0.39	**5.89** ± 0.28	**4.87** ± 0.31	**4.57** ± 0.22	**5.45** ± 0.29	**6.04** ± 0.37	**6.85** ± 0.40	**7.20** ± 0.49	**7.69** ± 0.35	**8.11** ± 0.46
LW (g)	**0.075** ± 0.007	**0.078** ± 0.004	**0.096** ± 0.006	**0.092** ± 0.005	**0.069** ± 0.004	**0.066** ± 0.005	**0.080** ± 0.005	**0.086** ± 0.006	**0.084** ± 0.006	**0.088** ± 0.005	**0.087** ± 0.005	**0.093** ± 0.005
SLA (cm^2g^{-1})	**69.0** ± 1.5	**60.1** ± 1.4	**65.6** ± 0.9	**64.8** ± 1.6	**70.5** ± 1.9	**74.7** ± 2.3	**68.4** ± 1.1	**71.0** ± 1.5	**82.5** ± 2.3	**81.0** ± 2.4	**89.8** ± 1.7	**87.6** ± 1.6
Total mass (g)	**7.02** ± 0.60	**6.32** ± 0.54	**7.54** ± 0.30	**6.24** ± 0.51	**5.50** ± 0.68	**4.80** ± 0.41	**8.84** ± 0.76	**8.40** ± 0.63	**8.30** ± 0.52	**7.86** ± 0.52	**9.93** ± 0.54	**9.27** ± 0.45
LA (cm^2)	**191** ± 21	**148** ± 15	**186** ± 9	**161** ± 15	**157** ± 20	**154** ± 18	**235** ± 28	**222** ± 25	**280** ± 21	**274** ± 15	**392** ± 21	**379** ± 25
S/R (gg^{-1})	**1.98** ± 0.19	**1.69** ± 0.13	**1.34** ± 0.10	**1.28** ± 0.12	**1.81** ± 0.17	**2.02** ± 0.21	**1.73** ± 0.16	**1.80** ± 0.20	**2.15** ± 0.16	**1.86** ± 0.13	**2.83** ± 0.15	**3.19** ± 0.34
LAR (cm^2g^{-1})	**27.3** ± 1.9	**23.1** ± 0.9	**24.9**± 1.1	**25.0** ± 1.4	**29.0** ± 1.4	**31.8** ± 1.6	**25.6** ± 1.8	**25.8** ± 1.8	**34.1** ± 1.2	**33.7** ± 1.2	**40.0** ± 0.9	**40.7** ± 1.4
LWR (gg^{-1})	**0.404** ± 0.024	**0.387**± 0.015	**0.380** ± 0.016	**0.387** ± 0.020	**0.407** ± 0.017	**0.416** ± 0.017	**0.378** ± 0.026	**0.363** ± 0.023	**0.415** ± 0.015	**0.407** ± 0.013	**0.448** ± 0.012	**0.464** ± 0.012
n	19	18	19	20	17	17	19	20	19	20	19	20

Table S4. Results of the PCA for normalized physiological variables (relativized as treated/control). In bold are evidenced the loading over 0.5. The principal factors represent the three factors with higher eigenvalues.

	Factor 1	**Factor 2**
A	**-0.556**	-0.283
GS	-0.272	-0.261
CI	0.364	-0.004
WUE	-0.300	-0.071
Fs	**-0.821**	0.150
Fm'	**-0.907**	0.034
Fo'	**-0.542**	-0.335
Fo	0.017	**-0.589**
Fm	-0.426	**-0.550**
Fv	**-0.503**	-0.486
Fv/Fm	**-0.614**	-0.017
ΦPSII	**-0.604**	-0.477
Fv'/Fm'	**-0.892**	0.316
Fq'	**-0.899**	-0.185
qP	-0.203	**-0.755**
NPQ	**0.687**	-0.478
(1-qP)/NPQ	**-0.585**	**0.633**
%D	**0.888**	-0.343
%P	**-0.604**	-0.477
%X	-0.481	**0.762**

Appendix S3. Supplemental figures S1-S4

Figure S1. Signs of injury observed on leaves of fumigated plants after 90 days of treatment. a) mottle stipple bronzing and necrosis signs b) chlorosis signs.

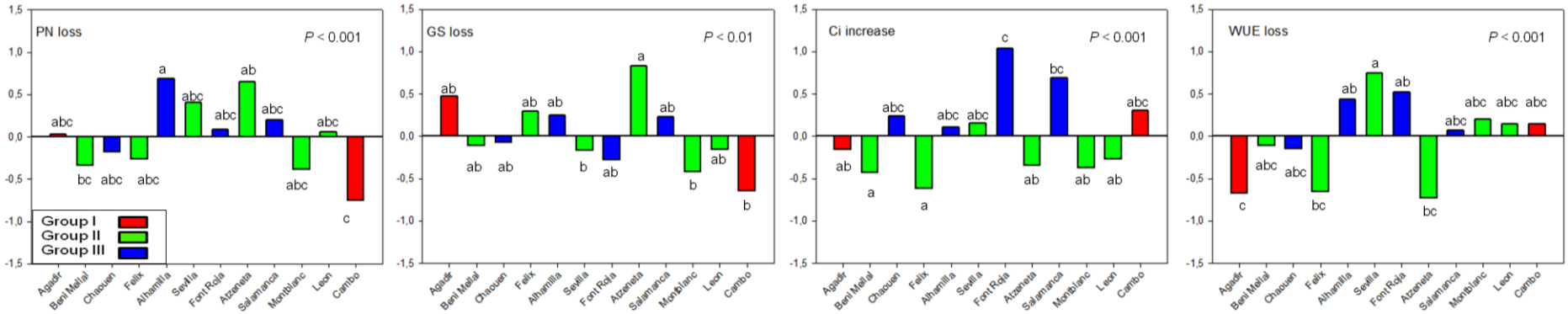

Figure S2. Normalized values of ozone effects relative Control to Treatment for the gas exchange variables.

Note: when the given variable displayed a loss of value, positive bars indicate losses above the year mean loss value, while negative bars indicate losses below the year mean loss value. On the other hand, when the studied variable displayed an increase of value, positive bars indicate increases above the year mean increase value, while negative bars indicate increases below the year mean increase value. Colours indicate the different groups of populations emerged from the PCA.

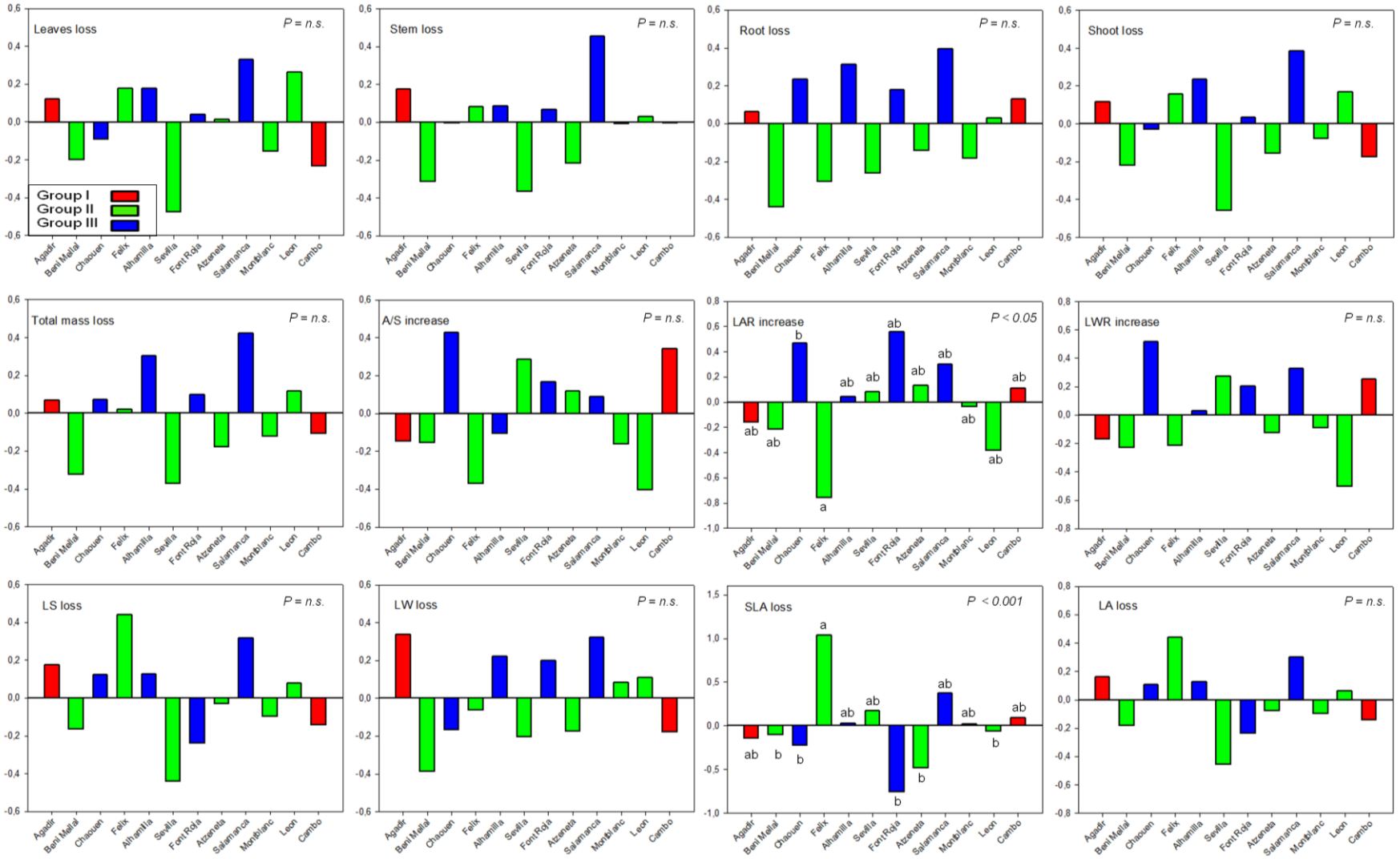

Figure S3. Normalized values of ozone effects relative Control to Treatment for biomass, structural variables and leaf traits.

Note: when the given variable displayed a loss of value, positive bars indicate losses above the year mean loss value, while negative bars indicate losses below the year mean loss value. On the other hand, when the studied variable displayed an increase of value, positive bars indicate increases above the year mean increase value, while negative bars indicate increases below the year mean increase value. Colours indicate the different groups of populations emerged from the PCA.

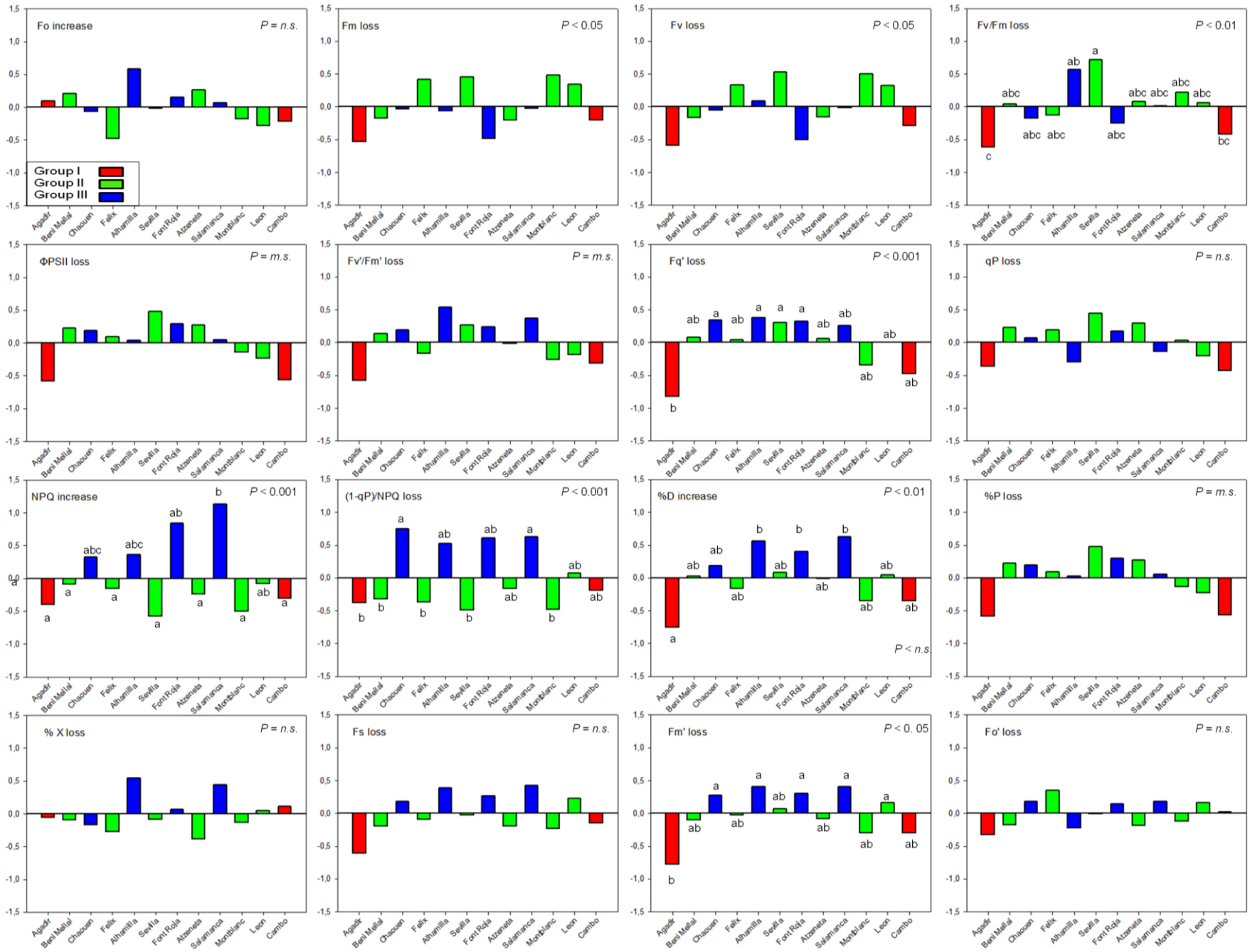

Figure S4. Normalized values of ozone effects relative Control to Treatment for the fluorescence variables.

Note: when the given variable displayed a loss of value, positive bars indicate losses above the year mean loss value, while negative bars indicate losses below the year mean loss value. On the other hand, when the studied variable displayed an increase of value, positive bars indicate increases above the year mean increase value, while negative bars indicate increases below the year mean increase value. Colours indicate the different groups of populations emerged from the PCA.

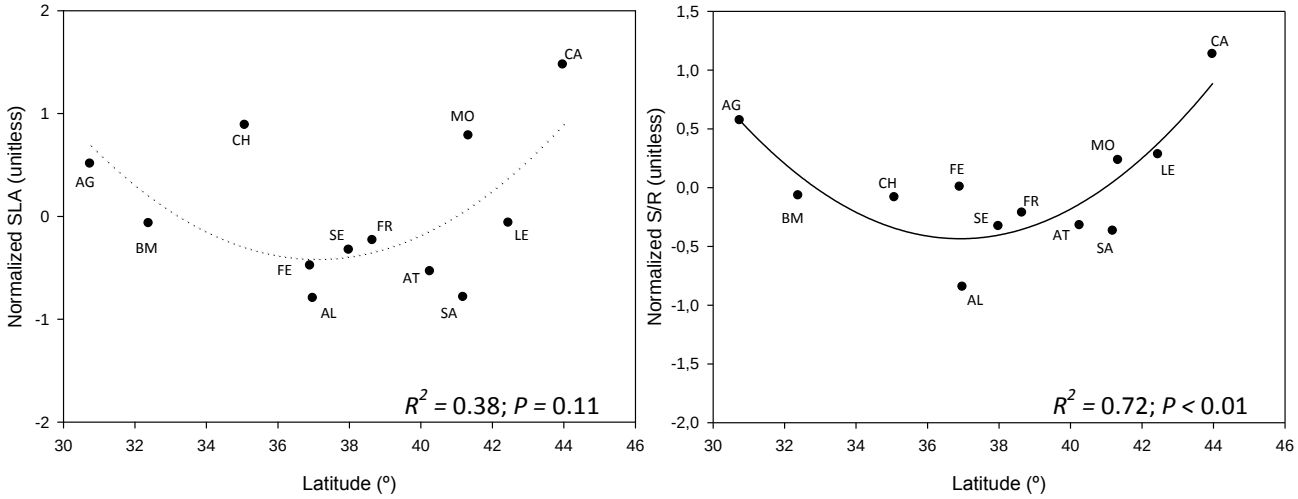

Figure S5. Relationships between latitude of origin and normalized data of SLA (a) and S/R (b) measured in Control seedlings grown in controlled chambers.

V. CAPÍTULO 4

Broad adaptive capacity on Photosystem II (PSII) in populations native to continental climates allows them to cope with both cold and warm seasonal temperature variability

Broad adaptive plasticity on Photosystem II (PSII) in populations native to continental climates allows them to cope with both cold and warm seasonal temperature variability

Ana García-Nogales, José I. Seco, Juan C. Linares, José Merino

1. ABSTRACT

Current climate changes are taking place likely faster than the dispersion ability of woody plant species does. Hence, a central issue in Biogeography is to investigate the extent to that populations within species hold enough adaptive capacity to cope with rapid climate change. Species' latitudinal gradient provides an experimental framework to investigate the intraspecific variation of plant functional traits, as well as to understand the implications of this variation to face changing environmental conditions. The intraspecific variability on photochemical performance has been still poorly examined, even though plant carbon balance might rely considerably on this physiological trait under a warming climate. We experimentally analyzed the photochemical response to seasonal thermal changes in seedlings of 12 *Quercus ilex* populations native to contrasted climatic habitats, including latitudinal range edges. We gathered acorns from natural stands sampled along the biogeographical distribution range and grew seedlings under common growth conditions for two years. Chlorophyll *a* fluorescence was assessed monthly during 18 months. High summer temperatures of the Mediterranean climate have greater impact on *Q. ilex* seedlings PSII performance than low winter temperatures. Although PSII performance showed plastic responses to seasonal temperature and radiation changes, our findings support also a strong relationship between climate of origin and PSII thermal tolerance. Specifically, the

populations native to colder climates (which also are subjected to the higher temperature oscillation) maintained higher photochemical performance (F_v/F_m, Φ_{PSII}, F_q') under both cold and warm conditions, whereas the populations from drier and warmer climates showed the highest chilling sensitivity (the highest heat dissipation) under winter conditions. Therefore, higher thermal fluctuations at site of origin could have promoted the selection of genotypes with higher adaptive plasticity to both chilling and warm temperatures, compared to populations native to provenances with more homogeneous climates.

Key-words: chlorophyll *a* fluorescence, down-regulation of photosynthesis, intraspecific variation, Mediterranean evergreen oak, morphological traits, non-photochemical quenching, phenotypic plasticity, photochemical quenching, Photosysthem II efficiency

2. INTRODUCTION

Increasing frequency and intensity of extreme climatic conditions, such as heat waves and frost events, is expected for the Mediterranean Basin as a result of Climate Change (IPCC 2014), which might modify the distribution of tree species (Linares & Tíscar, 2010). Current changes in environmental conditions may be faster than natural dispersal rates of long-living plant species (Jump *et al.*, 2009), as well as to overcome the pace of evolutionary adaptation processes (Bertrand *et al.*, 2011; Alberto *et al.*, 2013; Aranda *et al.*, 2015). Conservation of locally adapted ecotypes could provide a gene bank of traits conferring resistance against the potential extreme environmental conditions, as those expected for the Mediterranean Basin (Bussotti *et al.*, 2015). However, the intraspecific

variability on plant adaptive capacity to cope with rapid climate change through micro-evolutionary processes is still poorly understood.

Widely distributed tree species commonly exhibit both local differentiation and high levels of phenotypic plasticity. As regards locally differentiated adaptations of provenances, they allow to cope with the variety of environmental conditions throughout the species' distribution range (Abrams, 1994; Jump & Peñuelas; 2005; Bussotti *et al.*, 2015). Furthermore, phenotypic plasticity provides the ability of a given genotype to acclimate through modifications of its phenotype in response to a changing environment (Pigliucci, 2006). Thus, both genetic variability and phenotypic plasticity in functional traits should be taken into account to understand mechanisms linked to the capacity of adaptation to environmental changes (Meier & Leuschner, 2008; Rose *et al.*, 2009; Bresson *et al.*, 2011).

Patterns of intraspecific variation in functional traits provide valuable information about how species have evolved (Hoffmann & Sgrò 2011). Such information would be useful to predict future species' distribution shifts under a climate change scenario (Jump & Peñuelas, 2005). Furthermore, these changes are not likely to affect homogeneously across species' ranges; populations located at the edges of their distributions being especially sensitive to climate alterations (Linares & Tíscar, 2010; Matías & Jump, 2014). Understanding plant responses to different climatic drivers along wide latitudinal gradients, and the mechanisms underlying these responses, is thus particularly relevant to predict likely responses to climate change (Hoffmann & Sgrò 2011; Alberto *et al.*, 2013; Bussotti *et al.*, 2015). Nevertheless, to our knowledge, few studies have reported the intraspecific variation relative to physiological traits in response to thermal stress on the entire species' latitudinal range, including the range margins.

Climatic niche modelling approaches suffer from strong limitations on population's climatic requirements predictions; since it is still unclear whether populations within species show either plastic adaptive capacity to changing climatic conditions or local adaptation to their habitat constrains, or both and to what extent (Wiens & Graham, 2005; Pearman *et al*., 2008; Koehler *et al.,* 2012). As regards thermal tolerance, it have been hypothesised that species may have evolved conservatively if all populations have the same resistance either to freezing or warming, since adaptive plasticity can buffer the impact of changing conditions on populations (Alberto *et al.,* 2013). As a result, it would be expected little variation in climatic tolerance among populations. On the other hand, if populations were suited to local climates, they would show adaptive differentiation, as locally adapted ecotypes (Kawecki & Ebert, 2004). Even though high phenotypic plasticity tends to maximize fitness under changing environments, genetic differentiation might be favoured against plasticity in more constant environments due to the inherent costs of plasticity (Meier & Leuschner, 2008). Therefore, niche-based models may underestimate the adaptive potential of broadly distributed species, and consequently, they likely fail forecasting future distribution ranges (Morin *et al.,* 2007).

Wide species latitudinal gradients, as well as altitudinal ones, provide a suitable experimental model to study variation of functional plant traits in response to environmental conditions (Bresson *et al.,* 2011). According with that, a variety of studies have reported geographic variation in both cold (Rehfeldt *et al.,* 2002; Aranda *et al.* 2005; Cavender-Bares, 2007; Kreyling *et al.,* 2012) and drought/warm tolerance (Bruschi, 2010; Linares & Tíscar, 2010; Ramírez-Valiente *et al.*, 2009; Thiel *et al.*, 2014) on several tree species. These studies point out that local and regional variability

have broad implications about the ability of populations to face changing environmental scenarios.

Among the different morphological and physiological traits investigated in response to changing environmental conditions, Photosystem II (PSII) has been widely considered searching for ecophysiological plant adaptive responses (Bussotti *et al.*, 2015). The photosynthetic apparatus, especially the electron transport chain imbedded within the thylakoid membrane, is quite sensitive to cold and heat stress (Corcuera *et al.*, 2005b; Ducruet *et al.*, 2007). Alterations in membrane properties are a common consequence of temperature stress (Larcher, 2005). Thylakoid membranes are especially sensitive to heat because it induces an increase in membranes fluidity. As a result, thermotolerance of the photosynthetic apparatus may be inferred from the increase in basic fluorescence (i.e. the critical temperature), which is a useful tool to compare within or among species (Ghouil *et al.*, 2003; Daas *et al.*, 2008). On the other hand, the surplus of light energy in low temperature sunny days leads to down-regulation of photosynthesis (i.e. reduction of the potential efficiency of PSII and accumulation of carotenoids that increase heat dissipation) (Larcher, 2000; Aranda *et al.*, 2005); which could eventually result in chlorophyll degradation and leaf drop (Cavender-Bares, 2007).

Owing to the photochemical process is prone to be the first target under thermal stress (Larcher, 2000), chlorophyll *a* fluorescence has become a popular tool in evolutionary biology for assessing population differences in photosynthetic activity in response to contrasting environmental conditions (e.g. Aranda *et al.*, 2005; Corcuera *et al.*, 2011; Salmela *et al.*, 2011). An over-excitation of photosynthetic apparatus when photosynthetic rate is decreased by thermal stress may lead to photodamage caused by reactive oxygen species (ROS) (García-Plazaola & Becerril, 2000). Antioxidants and

antioxidant enzymes have a direct detoxification of ROS and reduce photooxidative damage (Faria *et al.,* 1996; García-Plazaola *et al.*, 1999a; García-Plazaola & Becerril, 2000). Hence, plants with different membrane configurations or those endowed constitutively with higher amount of defensive molecules might display an inherent ability to withstand the adverse effects of both low and high temperatures. Some studies have suggested that plants inhabiting stressful environments acquire cross-tolerance to climate constrains (García *et al.,* 1998; Larcher, 2005; Chapter 3).

Holm oak (*Quercus ilex* L.) is a broad-distributed tree species around the Mediterranean basin that undergoes high climatic variability throughout seasons, years and distribution range. As a result, is expected it might be adapted to tolerate a wide range of climatic conditions (García-Plazaola *et al.,* 2000); which make it a suitable study system to examine intraspecific differentiation and thermal acclimation capacity. Evidence for environment-driven differentiation in adaptive traits among *Q. ilex* populations has been found in earlier studies (García *et al.,* 1998; Gratani *et al.,* 2003; Pesoli *et al.,* 2003; Laureano *et al.,* 2008; Varone *et al.,* 2016; García-Nogales *et al.,* 2016). How the seasonal pattern of photochemical activity varies among *Q. ilex* populations native to diverse Mediterranean climate areas, growing under common-garden conditions, has not been examined yet in such great detail (but see Corcuera *et al.,* 2005b)

Here, we attempt to assess the plasticity in PSII photochemistry performance of *Q. ilex* under seasonal temperature changes, as well as to investigate the degree of intraspecific differentiation related to summer and winter growth temperatures and its relationship with the provenance climate. We hypothesised that: (i) PSII of *Q. ilex* will display high acclimation capacity to cope with seasonal temperature changes; (ii) populations will show intraspecific differentiation in response to changes in

temperature, providing evidence for local adaptation; and (iii) individuals native to colder climates will display the highest cold tolerance, whereas those native to driest and warmest climates will show the highest heat tolerance. Alternatively, plants from the edge range would exhibit lower phenotypic variation and high tolerance to extreme temperatures as a result of range limits-induced resistance to multiple stress factors.

3. MATERIAL AND METHODS

3.1. Seed collection, sowing and experimental set-up

Twelve populations of *Quercus ilex* subsp. *ballota* (Desf.) Samp. from natural stands were selected for the study. The latitudinal gradient was located in the western side of the Mediterranean Basin, covering central and boundaries populations across a wide range of climatic conditions (Table 1 and Fig. S1). The maximum mean summer temperature range from 33.5ºC in the southernmost population (Agadir) to 20.6 ºC in the northernmost population (Cambo); while the minimum mean winter temperature range -0.5ºC in León (a northern population) to 10.6ºC in Agadir. Local climate characterization for each population was obtained from the E-OBS v10.0 and CRU TS 3.22 datasets for the period 1950–2013 (KNMI Climate Explorer dataset: https://climexp.knmi.nl) (Oldenborgh & Burguers, 2005) following García-Nogales *et al.* (2016) methodology.

Acorns were collected from 20 different trees randomly chosen of each population (about 50 per tree) and taken to the laboratory. A subset of acorns were pooled by population and sown in 3 litre pots filled with a substrate (3:1 peat:perlite mixture). This mixture was supplemented with NPK fertilizer. Seedlings were grown

for two years under greenhouse conditions at Pablo de Olavide University, Seville (37°21'3N, 5°56'7W, 15 m.a.s.l.): day/night mean temperature of 19/11ºC, a mean RH of 62%, and a mean photon flux density of 300 µmol $m^{-2}s^{-1}$.

In order to test how populations respond to seasonal temperature changes, a common garden experiment was established outside the greenhouse with 20 seedlings randomly selected per population, placed facing south in a randomized block design. Plants were watered twice daily.

Table 1. Location and climatic details of the 12 provenances of *Q. ilex*. Mean annual temperature (T), mean maximum temperature in summer (Tmax), mean minimum temperature in winter (Tmin), annual mean rainfall (Pa), summer mean rainfall (Ps) corrected potential evapotranspiration (PET), water balance (Pa – PET) and thermal oscillation (Tmax – Tmin).

Code	***Population***	***Latitude (N)***	***Longitude (E-W)***	***Elevation (m.a.s.l.)***	***T (ºC)***	***Tmax (ºC)***	***Tmin (ºC)***	***Pa (mm)***	***Ps (mm)***	***PET (mm)***	***Water balance (mm)***	***Thermal oscillation (ºC)***
CA	Cambo	43º 58'	3º 48' E	610	10.9	20.6	2.4	658	105.2	653	5	18.2
LE	León	42° 27'	5° 58' W	871	10.8	29.7	-0.5	505	59.1	654	-149	30.3
MO	Montblanc	41º 20'	1º 07' E	882	11.3	24.3	2.8	532	90.5	662	-130	21.5
SA	Salamanca	41° 11'	5° 30' W	886	10.8	25.2	0.7	403	49.0	653	-250	24.5
AT	Atzeneta	40º 15'	0º 14' W	970	11.6	23.3	2.2	499	76.9	669	-169	21.2
FR	Font Roja	38º 39'	0º 32' W	1270	14.0	30.2	2.6	420	43.8	748	-328	27.6
SE	Sevilla	37° 59'	5° 57' W	347	17.1	33.5	5.7	574	19.8	890	-316	27.8
AL	Alhamilla	36º 59'	2º 23' W	1370	14.5	30.0	5.1	239	12.3	767	-528	24.9
FE	Felix	36º 54'	2º 40' W	1138	13.3	30.5	0.5	310	16.7	730	-420	30.0
CH	Chaouen	34° 58'	4° 42' W	1600	13.0	24.4	4.4	567	15.3	690	-123	20.3
BM	Beni Mellal	32° 23'	6° 12' W	850	17.2	27.1	8.8	443	18.9	849	-406	18.3
AG	Agadir	30° 45'	8° 44' W	1553	21.3	33.5	10.6	309	19.9	1136	-827	22.9

3.2. Chl a fluorescence measurements

For 18 months (from October 2012 to March 2014), chlorophyll *a* fluorescence measurements were performed monthly using a pulso-modulated portable fluorometer (FMS 2, Hansatech Instruments Ltd, Norfolk UK). Measurements were carried out on cloudless days (PPDF above 1000 µmol $m^{-2}s^{-1}$) at midday (11:00-13:00, solar time).

The determinations were made in intact full developed leaves located on the top third of the plant (similar leaf age among populations), and whenever possible, measurements were repeated on the same leaf along the study. Fluorescence emission was recorded at selected leaves during actinic illumination (F_s), followed by a saturating pulse that provides the maximum fluorescence during actinic illumination (F_m'). Minimal fluorescence from light-adapted leaf (F_o') was calculated as Oxborough & Baker (1997). Later, the sampled leaf was dark adapted for 30 minutes in order to the primary quinone acceptor of Photosystem II (Q_A) becomes maximally oxidized and thereby was obtained minimal fluorescence from dark-adapted leaf (F_o) upon excitation with a weak non-actinic beam. The maximum fluorescence (F_m) was recorded following a pulse of saturating light to maximally reduce Q_A. Variable fluorescence (F_v) was determined as the difference between F_m and F_o, and the maximum quantum yield of Q_A reduction was estimated as the ratio F_v/F_m. The actual quantum yield of Photosystem II (PSII) photochemistry, Φ_{PSII}, closely associated with the quantum yield of non-cyclic electron transport, was estimated from $(F_m' - F_s)/F_m'$ (Genty *et al.*1989). Calculation of non-photochemical quenching, related to dissipation of absorbed light energy (*NPQ*), was determined according to the equation $NPQ = F_m/F_m' - 1$ (Bilger & Björkman 1990). The coefficient of photochemical quenching q_P, related to the fraction of open PSII reaction centres, was estimated as $(F_m'-F_s)/(F_m'-F_o')$ (Schreiber *et al.* 1986). Photochemical quenching (F_q') was calculated as $F_m'-F_s$ (Baker, 2008).

At the end of the experiment, we collected leaves close to the sampled leaf in order to determine LS (mean fresh leaf-blade size [cm^2]), LW (mean leaf dry mass [g]) and SLA (fresh leaf surface to leaf dry mass [$cm^2\ g^{-1}$]). In addition, plants' height was recorded.

3.3. Statistical analyses

A General Linear Mixed Model (GLMM) of repeated measurements was performed for the main fluorescence variables, including *Month* and *Population* as fixed factors (as well as its interaction) and *Seedling* as random factor. The components of variance were obtained by restricted maximum likelihood (REML). A hierarchical analysis of variance was applied per month to partition the total variance accounted for inter-population or intra-population differences. Additional ANOVAs were performed per month in order to test the differences among populations. Data were examined to nonnormality and heterocedasticity and they were transformed when was necessary to meet the assumptions of ANOVA. When differences were relevant, a posteriori test was conducted (Tukey's Post hoc HSD) to make individual comparison at population level. Linear regression analyses were applied to test the relationship between climate or morphological variables and the photochemical performance of populations when they were exposed to different seasonal conditions along the experiment. Phenotypic plasticity for the main fluorescence variables was estimated as plasticity index (PI) following Ramírez-Valiente *et al.* (2015) (see Methods S1 in Supporting Information).

The mixed models, hierarchical analysis and linear regressions were conducted using R (R Development Core Team 2015). The rest of the analyses were performed in STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

4. RESULTS

Over the 18 months that experiment lasted, minimum temperatures ranged from 0.3 ºC in February 2013 to 17.7ºC in September 2013, while the maximum temperatures

ranged from 9.7ºC in December 2013 to 49.2ºC in July 2013 (Fig. 1a). July was the driest month because of both the extreme maximum temperatures and low relative humidity values (Fig. 1b).

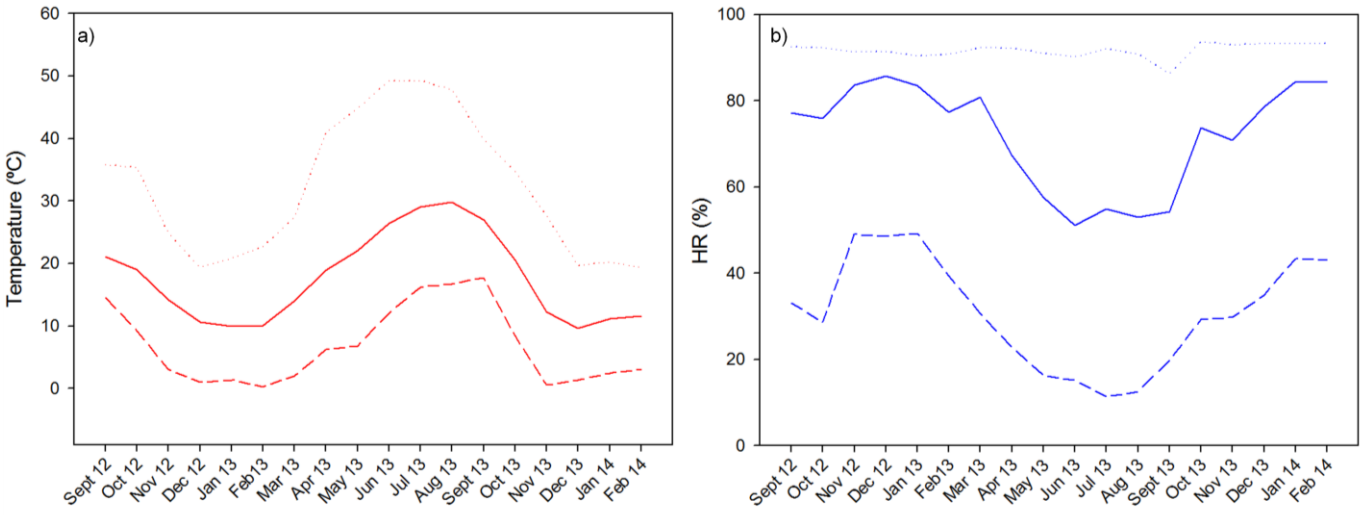

Figure 1.Seasonal variations in maximum (dot line), minimum (dash line) and mean (black line) monthly temperature (a) and relative humidity (b) at the experimental plot (Sevilla, Spain) during the 18-months study.

4.1. Photochemical performance response to seasonal changes

Seasonal changes (*Month*) and *Population* exerted a highly significant effect in all fluorescence variables considered (Table 2), the former having a major influence (see F Statistic). The generalized linear mixed model (GLMM) of repeated measures also showed a significant interaction between *Month* and *Population* for basal fluorescence (F_o), actual photochemical yield (Φ_{PSII}) and Photochemical Quenching (F_q') (see below).

October 2012 appeared to be the most favourable month recording the highest values of Photosystem II (PSII) photochemical performance (the highest values of Φ_{PSII}, F_q' and q_P; while the *NPQ* values were the lowest) in all populations (see Fig. 2 and Fig.

S2 in Supporting Information). With diminishing temperatures in February 2013, populations declined maximum photochemical yield of PSII (F_v/F_m), and especially actual PSII photochemical efficiency (Φ_{PSII}) and photochemical quenching (F_q'); attributable to both the increase in heat dissipation (*NPQ*) and the closure of the PSII reaction centres (q_P) (Fig. S2d-h). The highest drop in F_v/F_m was reached in summer months (July-August 2013), with minimum values ranging from 0.66 to 0.76, caused mainly by the increase in basal fluorescence (F_o). In addition, one of the highest *NPQ* increases (together with the increase of February 2014) was registered starting summer 2014, spanning values between 3.6 and 2.6.

Table 2. Repeated measures GLMM for the main fluorescence variables measured in both dark and light conditions. Month and Population (as well as its interaction) were included as fixed factors and Seedling as random factor.

Effect	numDF	denDF	F-value	p-value
Fo				
Month	15	3086	127.864	**<.0001**
Population	11	3086	9.635	**<.0001**
Month x Pop	165	3086	1.294	**0.0081**
Fm				
Month	15	3086	51.977	**<.0001**
Population	11	3086	12.081	**<.0001**
Month x Pop	165	3086	1.152	*0.0947*
Fv/Fm				
Month	15	3086	102.92	**<.0001**
Population	11	3086	7.22	**<.0001**
Month x Pop	165	3086	1.18	*0.059*
ΦPSII				
Month	15	3086	129.281	**<.0001**
Population	11	3086	12.706	**<.0001**
Month x Pop	165	3086	1.38	**0.0012**
Fq'				
Month	15	3084	120.9484	**<.0001**
Population	11	3084	14.8071	**<.0001**
Month x Pop	165	3084	1.2431	**0.0215**
NPQ				
Month	15	3082	31.875	**<.0001**
Population	11	3082	5.991	**<.0001**
Month x Pop	165	3082	1.003	0.4766
qP				
Month	15	3086	56.463	**<.0001**
Population	11	3086	5.16	**<.0001**
Month x Pop	165	3086	1.157	*0.0882*

Significant values ($P < 0.05$) are shown in bold and marginally significant values ($P < 0.1$) are shown in italics.

4.2. Variation among provenances

Population differences were more noticeable (Tables 3 and 4) in both the coldest month and the hottest month (February 2013 and July 2013, respectively; Fig. 1). A hierarchical ANOVA, with individual nested within population, showed that at chilling temperatures the percent of variance attributable to population was the highest, for F_q' being maximum (18%); followed by Φ_{PSII} (12.2%) and, in a lesser extent, F_v/F_m (11%) (Table 3). Furthermore, under summer conditions (July 2013), population variance was above 11% for the variables related to electronic transport chain, such as Φ_{PSII}, F_q' and q_P (Table 3). As indicated by the Plasticity index, in both the coldest and hottest month, the photochemical quenching (F_q') appears to be the most plastic fluorescence variable in this study, followed by Φ_{PSII} (Figure S3).

Fig. 2 displays the seasonal changes experienced per population in the main fluorescence variables throughout the experiment. A very significant population effect was detected in *NPQ* at the beginning of winter (December 2013) ($P < 0.001$, Table 4), where the African populations alongside the Montblanc population had the highest *NPQ* increase (Fig. 2g). PSII tolerance to chilling temperatures (represented by F_v/F_m, Φ_{PSII} and F_q' values) in the coldest month (February 2013) was significantly different among populations ($P < 0.01$, Table 4). Thus, both edges populations (Cambo and Agadir) exhibited the lowest values of Φ_{PSII} and F_q', whereas these variables reached the highest values in the Atzeneta and Font Roja populations, followed by León (Fig. 2d,e and S4d,e).

Table 3. Percentage (%) of the variance explained by random effects (Population, Seedling) for the main fluorescence variables, following a Hierarchical analysis of variance. Note that only the months and variables showing differences at inter-population level are displayed.

	Variance explained (%)									
Effects	***October 2012***	***December 2012***	***February 2013***	***April 2013***	***June 2013***	***July 2013***	***Late July 2013***	***August 2013***	***November 2013***	***February 2014***
Fo										
Population	-	9.2	-	-	-	7.8	3.5	5.2	13.5	9.9
Seedling	-	79.2	-	-	-	80.5	84.8	83.1	75.3	78.6
Residual	-	11.6	-	-	-	11.6	11.8	11.7	11.3	11.5
Fm										
Population	-	-	9.7	6.1	5.4	9.1	7.2	-	13.7	5.8
Seedling	-	-	78.8	82.3	82.8	79.4	81.1	-	75.0	82.5
Residual	-	-	11.5	11.7	11.7	11.6	11.7	-	11.2	11.7
Fv/Fm										
Population	-	7.7	11.0	-	2.2	-	5.0	-	7.3	-
Seedling	-	80.6	77.6	-	86.1	-	83.2	-	81.0	-
Residual	-	11.7	11.4	-	11.7	-	11.7	-	11.7	-
ΦPSII										
Population	4.8	9.4	12.2	-	3.7	12.7	7.7	-	-	-
Seedling	83.4	79.0	76.4	-	84.6	76.0	80.6	-	-	-
Residual	11.8	11.6	11.3	-	11.8	11.3	11.6	-	-	-
Fq'										
Population	-	14.0	18.0	7.2	10.4	11.1	8.6	-	-	-
Seedling	-	74.8	71.1	81.2	78.1	77.5	79.8	-	-	-
Residual	-	11.2	10.9	11.6	11.5	11.4	11.6	-	-	-
NPQ										
Population	-	10.1	-	-	-	-	-	-	-	9.8
Seedling	-	78.4	-	-	-	-	-	-	-	78.7
Residual	-	11.5	-	-	-	-	-	-	-	11.5
qP										
Population	9.0	3.5	-	-	-	12.6	-	-	-	6.5
Seedling	79.4	84.7	-	-	-	76.1	-	-	-	81.9
Residual	11.6	11.8	-	-	-	11.3	-	-	-	11.7

Table 4. ANOVA results for the effect of Population in the main fluorescence variables in each month along the experiment.

	Fo	***Fm***	***Fv/Fm***	***ΦPSII***	***Fq'***	***NPQ***	***qP***
October 2012	0.404	0.283	0.772	**0.034**	0.699	0.663	**0.003**
December 2012	**0.002**	0.788	**0.002**	**0.001**	**<0.001**	**<0.001**	**0.031**
February 2013	0.280	**0.004**	**0.002**	**0.001**	**<0.001**	0.223	*0.066*
April 2013	0.065	**0.038**	0.204	0.435	**0.014**	0.214	0.544
June 2013	0.075	**0.011**	**0.043**	**0.043**	**0.001**	0.459	0.689
July 2013	**0.005**	**0.001**	0.242	**0.008**	**0.049**	*0.074*	**<0.001**
Late July 2013	**0.015**	**0.008**	**0.035**	**0.007**	**0.005**	*0.081*	0.199
August 2013	**0.016**	*0.055*	0.643	*0.069*	0.135	0.471	0.150
November2013	**<0.001**	**<0.001**	**0.003**	0.471	0.219	0.782	0.369
February 2014	**0.001**	**0.023**	0.159	*0.066*	*0.074*	**0.001**	**0.015**

Significant values ($P < 0.05$) are shown in bold and marginally significant values ($P < 0.1$) are shown in italics

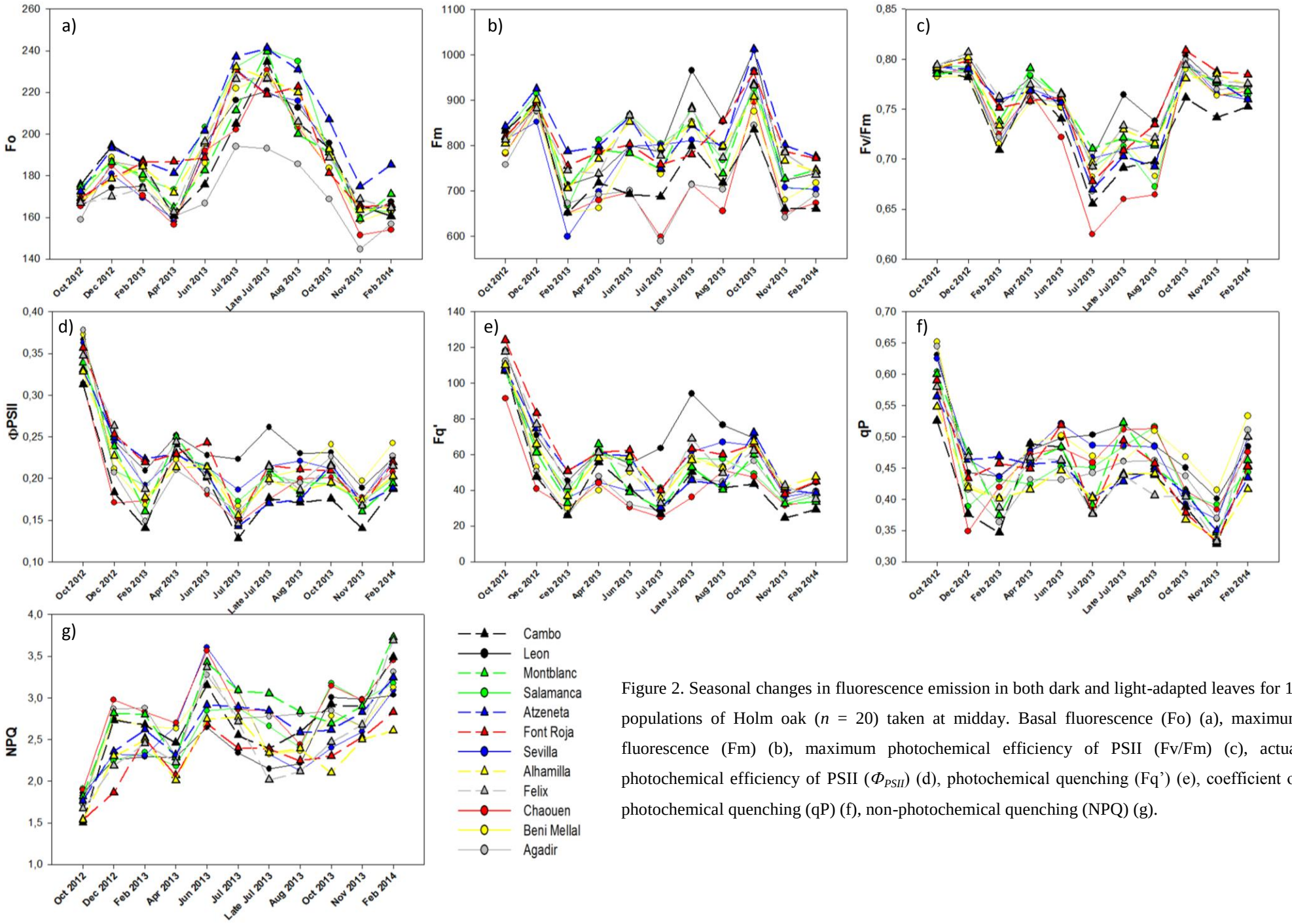

Figure 2. Seasonal changes in fluorescence emission in both dark and light-adapted leaves for 12 populations of Holm oak (n = 20) taken at midday. Basal fluorescence (Fo) (a), maximum fluorescence (Fm) (b), maximum photochemical efficiency of PSII (Fv/Fm) (c), actual photochemical efficiency of PSII (Φ_{PSII}) (d), photochemical quenching (Fq') (e), coefficient of photochemical quenching (qP) (f), non-photochemical quenching (NPQ) (g).

Regarding summer conditions, the southernmost population (Agadir) showed significantly lower values of basal fluorescence (F_o) under the extremely high temperatures reached in July 2013 with respect to the other populations (193 vs. 230 Figure S4a); with the slightest increment of this variable (roughly 21%, Fig. 2a) regarding its initial value (October 2012). This result indicates less degree of chronic photoinhibition or higher thermotolerance. By contrast, the northern populations (Cambo, Montblanc, Salamanca and Atzeneta), except León, exhibited the highest values of F_o in July 2013 (Fig. S4a). This resulted in the above-mentioned significant effect of the interaction between *Month* x *Population* on F_o ($P < 0.01$, Table 2). On the other hand, regarding the photochemical performance, the León population registered both significantly higher values and the lowest decrease of photochemical efficiency (F_v/F_m and Φ_{PSII}), as well as of F_q', when the seedlings were exposed to the highest temperatures in late July 2013 (Fig. 2c-e). On the contrary, the Chaouen population experienced the significantly highest drop in maximum PSII yield (F_v/F_m and Φ_{PSII}), and also F_q', accompanied by the Atzeneta and Cambo populations (Fig. 2c-e and S4c-e). Hence, the significant *Month* x *Population* interactions for Φ_{PSII} and F_q' ($P < 0.05$, Table 2) can be explained by the higher photochemical performance exhibited by León population at the hottest months compared with the rest of populations (Fig. 2d-e).

4.3. Trade-offs between temperature tolerance, morphology and climatic origin

Figs. 3 and 4 display the most significant linear regressions among fluorescence variables, leaf traits and climate (for further regressions see Tables S2-S5 in Supporting Information). Leaf traits were significantly correlated with fluorescence variables at mild, cold and warm months ($P < 0.01$, Fig. 3a-d). The leaf size (i.e. leaf-blade size [LS], and leaf weight [LW]) was negatively associated with maximum fluorescence (F_m), as well as with photochemical quenching (F_q'). Besides, the heat dissipation,

represented by non-photochemical quenching (*NPQ*), showed a significant and positive relationship with leaf-blade size. All together indicate that populations characterized by smaller leaves are able to catch and trap more amount of energy per unit area than those with larger leaves. On the other hand, with reference to climate, we found a significant and positive correlation between thermal oscillation at site of origin and mean population PSII yields (F_v/F_m and Φ_{PSII}) ($P < 0.01$; Fig. 3e and Table S4). Besides, seedlings native to habitats with high levels of aridity (i.e. high values of PET) displayed low F_o values in late July 2013 ($P < 0.001$; Fig. 3f).

Regarding the response of PSII to Mediterranean climate extreme temperatures, significant relationships were detected with climate characteristics across the 12 *Q. ilex* populations (Fig. 4 and Table S5). Populations native to habitats characterized by low winter temperatures had higher tolerance (in terms of actual PSII efficiency, Φ_{PSII}) to cold temperatures (Fig. 4a). Furthermore, populations characterized by higher thermal oscillation along the year had higher tolerance to warm temperatures (Fig. 4b), compared with populations less thermally fluctuating. On the other hand, the aridity of the source populations significantly predicted the basal fluorescence (F_o) increase depending on the month (Fig. 4c-d). Hence, the driest provenance showed the highest increase in F_o values at February 2013 and the lowest increase at late July 2013 (around 16% and 21%, respectively, Fig. 4c-d). On the contrary, the wetter provenances showed the opposite behavior. Besides, we found evidence for a trade-off between morphological traits and PSII cold tolerance. Seedling height and leaf size were negatively correlated with the decrease of both PSII photochemical yields (F_v/F_m, Φ_{PSII}, Fig. 4e-g), i.e. either larger seedlings or larger leaves had higher sensitivity to cold temperatures than the smaller ones.

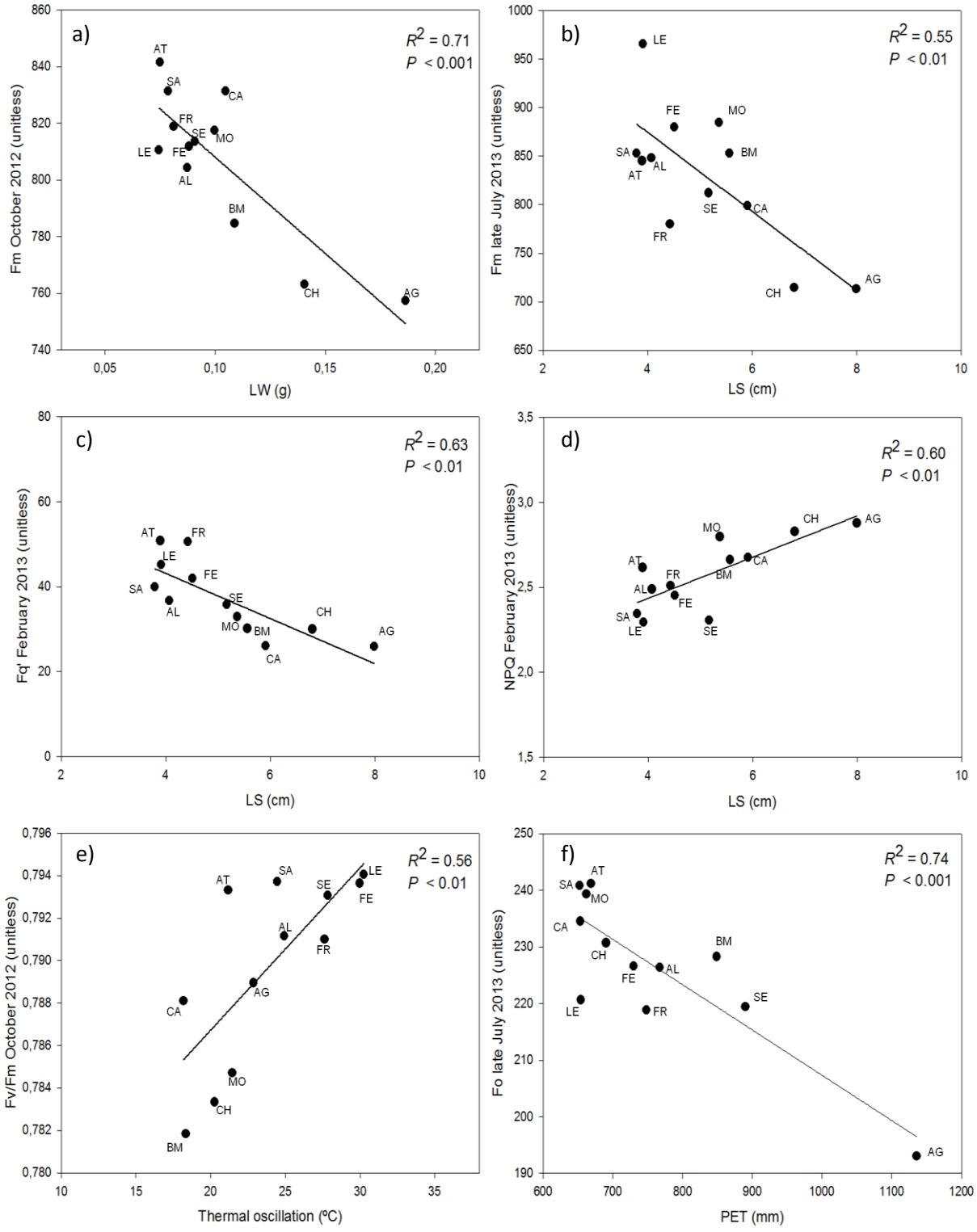

Figure 3. Trade-offs between fluorescence variables measured in the most significant months and both mean climatic data of origin and mean morphological traits characteristic of source provenance from 12 *Q. ilex* populations. Relationships are shown for: mean Fm values measured in October 2012 and mean individual leaf weight (LW) (a), mean Fm values measured in late July 2013 and mean leaf surface (LS) (b); mean Fq' values measured in February 2013 and LS (c); mean NPQ values measured in February 2013 and LS (d); mean Fv/Fm values measured in October 2012 and thermal oscillation (e); mean Fo values measured in July 2013 and annual potential evapotranspiration (PET) (f). Least squares fitted lines are shown for significant relationships ($P < 0.05$).

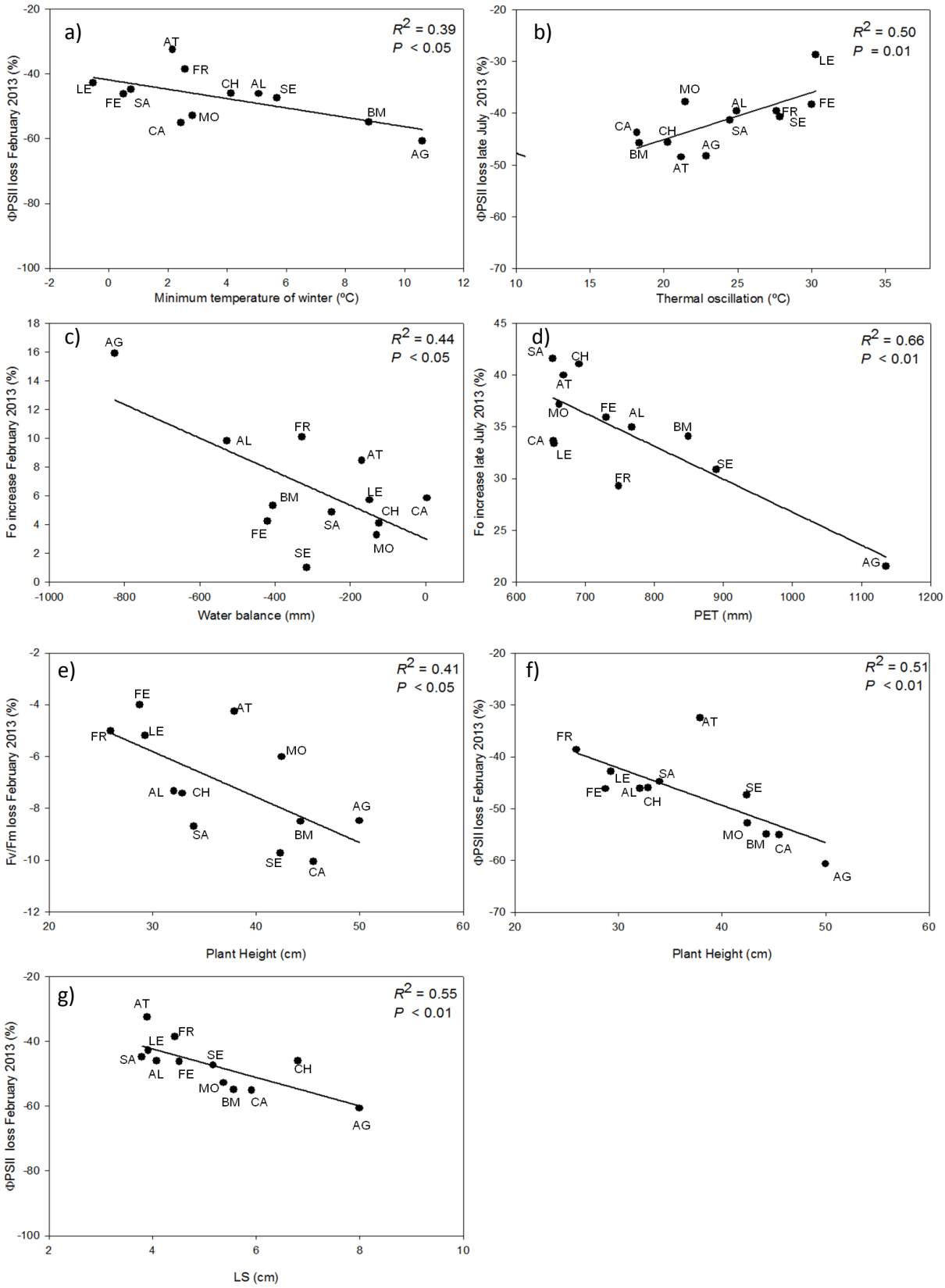

Figure 4. Relationships between fluorescence variables changes in February and July and both mean climatic data in the source of origin and mean morphological traits from twelve *Q. ilex* populations. Minimum temperature of winter predicts mean ΦPSII loss in February (a); thermal oscillation predicts mean ΦPSII loss in July 2013 (b). Water balance (Pa-PET) predicts mean Fo increase in February 2013(c); PET predicts mean Fo increase in July 2013 (d). Mean plant height predicts mean Fv/Fm (e) and ΦPSII (f) losses in February 2013. Mean leaf surface (LS) predicts mean ΦPSII loss in February 2013 (g). Least squares fitted lines are shown for significant relationships (P < 0.05).

5. DISCUSSION

The way to that some species are able to inhabit a broad range of climatic conditions remains a timely and relevant biogeographical subject, and is an important challenge for climate change research (Bennet *et al.* 2016). Usually, this broad range relies on different strategies, such as genetic diversity, ecotypic differentiation and/or phenotypic plasticity in morphological and physiological traits. Photosystem II (PSII) performance has been one of the functional traits largely used to screen for adapted genotypes in common gardens works (Bussotti *et al.,* 2015). In our study, we found that PSII of *Q. ilex* seedlings have a wide adaptive capacity to seasonal temperature changes, experienced over a period of 18 months. The high summer temperatures had a greater impact on PSII performance than low temperatures during the winter (see below). Furthermore, our results evidence significant differences in cold and warm sensitivity among *Q. ilex* populations, across a wide latitudinal gradient, from France to Morocco.

5.1. *Acclimation capacity to seasonal changes*

Seasonal change in temperature (*Month*) contributed to the phenotypic variation in photochemical variables in a greater extent than *Population* (Table 2), consistent with the findings of several common garden studies on tree species (Meier & Leuschman, 2008; Bresson *et al.,* 2011; Corcuera *et al.,* 2011). Most of the variation in photochemical variables was attributed to variance within-population (above 70 % in all fluorescence variables), reflecting substantial diversity and phenotypic plasticity at population level (Table 3 and Fig. S3). Our results support that phenotypic plasticity is an important strategy in *Q. ilex* to cope with environmental heterogeneity, which, in addition, could potentially facilitate rapid adaptation under a climate change scenario (Bresson *et al.*, 2011).

The highest values of photochemical yields (F_v/F_m, Φ_{PSII}) and photochemical quenching (F_q') were measured in October 2012 for all *Q. ilex* populations, which was in agreement with previous determinations of actual capacity of PSII photochemistry performed in *Q. ilex* (Larcher, 2000). Data from this author revealed that below 15ºC and above 35ºC leaves of this species suffer from thermal stress. In our study, the highest values of F_v/F_m and Φ_{PSII} could be explained by the more favourable temperatures of October 2012. The differences regarding October 2013 could be attributed to leaf senescence.

Under winter conditions, specifically in February 2013, an overall decrease in photochemical performance was recorded (Fig. S2d-f), probably because low temperatures reduce the mobility of the plastoquinone in thylakoid membranes; according to previous studies on *Q. ilex* (Corcuera *et al*., 2005a, b). This decrease could be attributable to the so-called down-regulation of photosynthesis rather than to damage in the photosynthetic apparatus, owing to the decrease in maximum PSII efficiency (F_v/F_m) was achieved by a strong decline in maximal fluorescence values (F_m) combined with a slight increase of basal fluorescence (F_o) (Demmig-Adams, 1990). Down-regulation of photosynthesis usually is activated in order to avoid over-excitation in the photosynthetic apparatus under limiting CO_2 assimilation, as a result of the slowing down of the enzymatic processes caused by cooler temperatures while light absorption continues at PSII (Corcuera *et al.*, 2005b). In our case, the excess energy absorbed by PSII antenna complex is being released through non-photochemical dissipation, as is confirmed by the *NPQ* increase (Fig. S2h). Acclimation to cold temperatures in overwintering evergreen species, including *Q. ilex*, has been linked to increases in antioxidants and xanthophylls pigments, as well as changes in the

composition of PSII antenna (García-Plazaola *et al.*, 1999b; Cavender-Bares *et al.*, 2005), resulting in an increase of *NPQ* values.

In Mediterranean climate regions, summer soil and atmospheric water deficits are the main limiting factors for photosynthesis and growth (Faria *et al.*, 1998; Larcher, 2000). In our study, plants did not experience water deficits because they were watered regularly; however, the high temperature conditions during summer at common garden (Fig. 1) could result in a strong VPD increase, which likely contribute to stomatal closure, causing a reduction of CO_2 assimilation (Faria *et al.*, 1996). When carbon assimilation decreases during summer, chloroplasts may experience an excess of light energy that cannot be driven to photosynthetic electron transport chain; which may generate reactive oxygen species (ROS) resulting in photodamage (Demmig-Adams & Adams, 1996). So, it appears that in order to reduce the possibility of further damage to the PSII reaction centres, *Q. ilex* seedlings react following the down-regulation strategy: firstly, increasing heat dissipation directly in the chlorophyll/carotenoid-binding antenna complexes of PSII (*NPQ* increases). Secondly, diminishing the capacity for light harvesting (F_v/F_m drop) (Fig. S2h and S2d, respectively), probably by changes in chlorophyll content (Krause, 1988; Munné Bosch & Alegre, 2000).

The F_v/F_m drop observed in summer 2013 was even deeper than registered in winter, in contrast to other seasonal studies on *Quercus* species (Aranda *et al.*, 2005; Corcuera *et al.*, 2005b). The summer decline was caused especially by the strong increase in F_o values (Fig. S2a), which denotes an inactivation of PSII reaction centres due to thylakoid instability, damage to PSII, and uncoupling between antenna and PSII cores (Krause, 1988). Therefore, severe damage to photosystems is occurring only under high temperatures, as was evidenced by the steep increase of F_o compared with initial values (34.4% in late July vs. 6.5% in February), while F_m values remains in

similar values than those from October 2012 (see Fig. S2b). This result has been usually registered under high temperatures in several species (Ghouil *et al.*, 2003; Ducruet *et al.*, 2007; Daas *et al.*, 2008).

In late July 2013, saplings seem to have developed heat hardening, as suggests the slight recovery in photochemical yields (Fig. S2d,e). Under long-term summer warm, *Q. ilex* showed a great heat dissipation capacity (showing maximum values in June 2013), suggesting that this species is able to increase *NPQ* in response to warm temperatures to avoid further photodamage at PSII under stressful summer conditions. Our results agree with the increase in the pool of de-epoxidated xanthophylls (closely related to *NPQ* increase) reported in the *Q. ilex* under drought treatment (Peguero-Pina *et al.*, 2009).

5.2. Intraspecific strategies to cope with thermal stress

Q. ilex seedlings showed significant differentiation in temperature sensitivity, related to their provenance, as expected from their wide distribution range. Population variation was displayed mainly in February and July 2013 (Table 3) (the coldest and hottest month, respectively), according to earlier studies suggesting that population differentiation arise mainly under adverse conditions (Aranda *et al.*, 2005; Corcuera *et al.*, 2011; Andivia *et al.*, 2012a; Ramírez-Valiente *et al.*, 2015). We found that populations from geographic regions with lower minimum temperatures were more tolerant to cold stress in winter, thereby suggesting local adaptation to chilling conditions (Aranda *et al.*, 2005; Cavender-Bares, 2007; Andivia *et al.*, 2012a; Kreyling *et al.*, 2012). However, it is noticeable that, opposite to expected, the populations native to colder regions also had better photochemical performance in response to warm temperatures, compared to those native to xeric regions (see below).

Warm growth conditions provided the most significant evidence of intraspecific differentiation (Figs. 1a and 2). The sharpest drop in photochemical capacity (F_v/F_m) in such period was observed in seedlings from a population native to climatically mild area (Chaouen), while those from coldest provenance (León) exhibited the highest warming tolerance under extremely high temperatures (around 49ºC, Fig. 2c). León seems to be the most heat tolerant population because, in spite of the summer stress conditions, is able to hold up the highest maximum photochemical efficiency (F_v/F_m), as well as the higher values of light utilisation for photochemistry (Φ_{PSII} and F_q') by maintaining low values of thermal dissipation (*NPQ*) (Fig. 2c-g). On the other hand, Chaouen strongly down-regulates the delivery of excitation energy into the electron-transport chain (F_v/F_m) together with a likely photodamage, as was indicated by high F_o values regarding low F_m ones (Fig. 2a,b).

It is accepted that plants may cope with different environmental stressors through similar responses at the cellular and molecular level, when these stressors trigger similar signal chains (García *et al.*, 1998; Kreyling *et al.*, 2012). Since the underlying mechanisms for drought and frost tolerance involve the reduction of cell dehydration, as well as the preservation of membrane integrity (e.g. intracellular sugar production, synthesis of heat shock proteins, membrane lipid composition; see Raison & Berry, 1979; Larcher, 2000 and 2005; Gimeno *et al.*, 2009; Andivia *et al.*, 2012a), it is not surprising that the colder provenance (León) exhibits the higher thermotolerance to both winter and summer stress, as assessed by photochemical performance. This result suggest that common constitutive mechanisms could be involved in protection against damage by both high and low extreme temperatures (see also Gimeno *et al.* 2009). In short, the observed differences in photochemical capacity under high temperatures could be due to convergent cold and warm hardiness.

During the summer 2013, maximum temperatures reached 49ºC, which is close to critical temperature (assessed by increases in basal fluorescence) for Mediterranean species, including *Q. ilex* (Méthy *et al.*, 1997; Larcher, 2000). Such temperature could have showed up the highly significant differences in thermotolerance among populations. Tolerance of the photosynthetic apparatus to high temperatures is thought to be mainly related to the thermostability of PSII photochemistry, since heat stress may increase both thylakoids membrane fluidity and protein denaturalization (Ghouil *et al.*, 2003). Regarding the basal fluorescence (F_o) increase during summer, our study suggests different critical temperature among populations related to the aridity of its geographical origin. Thus, a negative significant relationship was found between the populations' basal fluorescence emission and PET in source habitats; with the southernmost population (Agadir) characterized by the driest conditions (i.e. the highest PET value, 1136 mm) exhibiting both the lowest F_o value (Fig. 3f) and the slightest increase in F_o under summer stress (Fig. 4d). Furthermore, there is some evidence of higher concentration in membrane stabilizers in the African populations, such as carotenoids (Table S6), which may counteract the heat-induced hyperfluidity (Ducruet *et al.*, 2007). Therefore, it could be accepted that basal fluorescence (a surrogate of thylakoid membranes fluidity) is submitted to selection through climatic constrains related to drought.

Carotenoids are pigments highly related to the endurance of the combination of low temperature and high irradiation in winter (García-Plazaola *et al.*, 1999b). The above-mentioned higher photoprotective pigments concentration in the African populations is in accordance with the significant population effect detected in the increase in *NPQ* values under cold temperatures. North Africa populations, along with Montblanc and Cambo populations, displayed the highest *NPQ* increase starting winter

2012/2013 (Fig. 2g), suggesting that short-term exposure to cold temperatures triggers a steeper increase in heat dissipation in populations characterised by cold-sensitive leaves and endowed with higher amount of carotenoids than in the rest of populations. Although, the carotenoids content of Cambo and Montblanc populations has not been analysed yet, such populations are characterized by low photochemical efficiency (Φ_{PSII} and F_q'); therefore, one would expect that the absorbed light excess were delivered by heat dissipation in a greater extent.

5.3. Explaining trade-offs between morphology, climate and the thermal seasonal stress response

Cold or warm hardiness in some tree species has been shown to be genetically inherited (Salmela *et al.*, 2011; Andivia *et al.*, 2012a), as well as maternal effect can have some temporary effect on a given trait (Ramírez-Valiente *et al.*, 2009). In our study on potted seedlings, the intrinsic population characteristics related to PSII stability seem to be of greater importance than the maternal effect, since the acorn mass was unrelated to neither the cold nor warm tolerance.

Morphological data support the differentiation among populations, as edge populations (Agadir and Cambo) allocate a greater proportion of its resources to shoots and have large leaves (Table S1), accordingly to previous studies on the same populations (Chapters 2 & 3). Besides, the populations from climates with colder winters (León, Felix, Salamanca, Atzeneta and Font Roja) have small seedlings with small leaves (Fig. S5a-c), and have also a greater extreme temperature tolerance than those from less colder climates, as indicated by their lower decrease in photochemical performance (Φ_{PSII}) in both winter and summer (Fig. 4a and Table S5). These results agree with previous studies showing that temperature is positively correlated to growth rate (Rehfeldt *et al.*, 2001; Green, 2007; Gimeno *et al.*, 2009; Savage & Cavender-

Bares, 2013), which is also positively related to leaf area (Moles *et al.*, 2014). The significant relationship found here between leaf size and minimum temperature of winter at source populations suggests that the higher tolerance to cold temperatures is attributable partly to populations characterized by smaller leaves (Fig. 4g). As heat dissipation was low for populations with small leaf size (Fig. 3d), LS of the coldest provenance (León) seems to be more likely related to thermal tolerance than to the photoprotective mechanisms (*NPQ*), according with García-Plazaola & Becerril (2000). These authors stated that drought tolerance was based on morphological characteristics to a greater extent than on xanthophylls-meditated photoprotective responses.

We observed that the populations with smaller leaves exhibit a more efficient photosynthetic apparatus (Fig. 3a-d) and appear to be able to acclimate to both cold and warm temperatures better than the less efficient ones. Thus, since the amount of electrons flowing through the electron transport chain (F_q') has been shown as a proxy for photosynthetic rate in *Q. ilex* seedlings (Chapter 3), populations that, in average, exhibited higher F_q' values (León, Felix, and Font Roja, Fig. 2e) seem to be more tolerant to winter and summer temperatures (Fig. S4d,e). Thus, if cold or heat hardiness are mainly energy-dependent processes, it would be expected that a high photosynthetic performance may render the accumulation of soluble carbohydrates, fatty acids and other compounds which have important roles in cell membrane maintenance and stability; improving the plants' thermal tolerance (Andivia *et al.*, 2012b). Overall, in the populations characterized by more severe winters and more fluctuating temperatures along the year, individuals seem to have been selected for smaller leaves, photosynthetically more efficient, and with higher investment in extreme temperature tolerance strategies, as discussed above.

Reduced growth in cold climates has been previously reported for several species (Koehler *et al.*, 2012; Savage & Cavender-Bares, 2013), including *Q. ilex* (García *et al*., 1998; but see Laureano *et al*., 2016); and there are likely multiple factors contributing to this relationship. In particular, the present study supports the explanation that resource allocation to cold tolerance limits allocation to growth (Fig. 4e-f), in spite of the high photochemical efficiency reported in the present study. Several biochemical changes occur during cold acclimation that confer freezing tolerance, including increase in both cellular lipid and water-soluble solutes, chemical and conformational changes in cell membranes; all which require reallocation of carbon and nutrients, resulting in reduced growth (Savage & Cavender-Bares, 2013). Therefore, resource investment in traits for extreme temperature tolerance is hypothesized to cause more conservative growth (Koehler *et al.* 2012). The lower seedling height and leaf size in source locations with colder winters seem to support this hypothesis (Table S1). By the opposite, the largest seedling size populations, specifically the edge populations (Agadir and Cambo, Table S1), appear to be among the most sensitive to extreme temperatures reached in both winter and summer 2013 in terms of photochemical efficiency (Figs. 2c-e and S4c-e). Such populations, characterized by resource limitation at origin, possibly have evolved to invest in growth more rapidly than others, in order to take advantage under favourable year growth conditions (Koehler *et al.*, 2012; Savage & Cavender-Bares, 2013). As a result, they compensate their low photochemical efficiency with high allocation to the aerial fraction; resulting in the biggest individuals (Chapter 2). Therefore, the edge populations would invest in a lesser extent in chilling or warming acclimation than other populations. Our data contrast with other studies that expect a more fixed growth rate at the margin of a species' range as a consequence of a more conservative adaptive strategy (Green, 2007).

The invariability of functional traits is commonly stronger at the margins of the species range (Sanchez-Salguero *et al.*, 2015) owing to both biotic and abiotic stress usually increases at the species' distribution limits (Vergeer & Kunin, 2013). Following the biogeographical theories, the edge populations might be inherently issued with higher investment in stress defense endowments, compared to core populations, because of the extreme environmental conditions at the range edges; as has been suggested in previous studies (García *et al.*, 1998; Laureano *et al.*, 2016; García-Nogales *et al.*, 2016, Chapter 3). Contrary to our expectations, the putative constitutive defense investments in *Q. ilex* do not provide a reliable explanation for the different thermal tolerance among populations. Therefore, it is suggested that the thermal tolerance is probably more related to specific mechanisms linked to temperature rather than to putative constitutive general defense endowments. Indeed, the mechanisms conferring resistance to extreme temperatures are not straightforward; as the provenances from harsh winters habitats display high tolerance to winter cold temperatures, which also confers resistance to summer high temperatures. Nonetheless, the provenances native to warm summers habitats seem not to be endowed with resistance to cold temperatures.

León and Felix populations (even though are not located at range edges) are characterized by the highest climate variability in their origin habitats (higher differences between maximum and minimum temperatures, Table 1), which poses a source of abiotic stress that populations have to cope with (Morin & Chuine, 2006). Such populations seem to be under a stronger selection process than populations on more favourable sites, and this might have conferred a high adaptive plasticity in PSII performance to withstand both high and low temperatures (Meier & Leuschner, 2008); which is more noticeable in the León population. Indeed, the above-mentioned potential trade-off between growth in plants and increased tolerance to abiotic stress supports this

explanation, since the smallest seedlings were those from León and Felix (plus Font Roja). The simultaneous tolerance to warm and cold conditions is a universal trait for Mediterranean climate species, and has paramount importance for *Q. ilex* populations inhabiting continental areas, where they are under strong seasonal thermal oscillations. Paradoxically, the northernmost population (Cambo) is not the best performing in winter, displaying low photochemical performance in the current study. This result could be explained because this northernmost population experiences mild winters at source provenance (Table 1) due to the Mediterranean Sea influence (Chapter 2). On the other hand, the southernmost population (Agadir) showed the highest heat tolerance in terms of thylakoid membrane stability (lower F_o increase), revealing strong local adaptation to the most severe warm and xeric conditions in its origin.

5.4. Conclusions

Our results revealed high thermal tolerance and acclimation capacity to both warm and cold temperatures in PSII of *Q. ilex* seedlings, as has been reported for other tree species (Aranda *et al.*, 2005; Corcuera *et al*., 2005b and 2011), including *Q. ilex*. Seasonal temperature changes affected overall photochemical performance of plants from all provenances, suggesting significant phenotypic plasticity in PSII performance (fluorescence variables), and besides, a clear role of local adaptation as regards the PSII response to contrasting climatic conditions.

Thus, our findings indicate that there is a relationship between the local climate of provenance and the PSII thermal tolerance, while this relationship depends on the variable considered. Specifically, the populations from colder climates maintained higher photochemical performance (F_v/F_m, Φ_{PSII}, F_q') under both cold and warm conditions, whereas the populations originating from drier and warmer climates showed the highest chilling sensitivity (the highest heat dissipation) under winter conditions.

Besides, Agadir, the southernmost population exhibited the greatest stability of the thylakoid membrane (regarding F_o values) in summer, indicating that basal fluorescence could be a trait subjected to evolution. The ability to acclimate and express higher chilling/warming tolerance of colder populations was associated with low growth rates and a reduced leaf size, suggesting lower competitive capacity. Our results indicate that, at colder locations, *Q. ilex* have been selected to invest in cold hardiness, rather than higher growth rates and larger seedlings. The León case highlights that populations subjected to continental climates display the highest resistance to chilling and warm temperatures. By the contrary, the resistance to high temperatures (Agadir) does not seem to confer low temperature tolerance.

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7. SUPPORTING INFORMATION

Methods S1. Plasticity index (PI) for fluorescence variables were calculated as:

$$PI = \frac{mean(env1) - mean(env2)}{mean(env1) + mean(env2)}$$

Where *mean(env1)* is the average value for a given fluorescence variable in October 2012, the reference month; while *mean(env2)* is the average value in February 2013 or late July 2013, the coldest and the hottest month, respectively.

Table S1. ANOVA results for the effect of Population in leaves traits.

	LS (cm^2)	LW (g)	SLA ($cm^2\ g^{-1}$)	Plant height (cm)	n
Cambo	5.9 ± 0.5 cde	0.105 ± 0.009 abcd	59.2 ± 3.7 b	45.5 ± 2.2 f	19
Leon	3.9 ± 0.3 a	0.074 ± 0.007 ab	55.1 ± 2.4 ab	29.3 ± 2.5 ab	16
Montblanc	5.4 ± 0.3 bcde	0.100 ± 0.006 abcd	54.9 ± 1.6 ab	42.5 ± 2.1 ef	20
Salamanca	3.8 ± 0.3 a	0.079 ± 0.007 ab	49.3 ± 2.0 ab	34.0 ± 2.1 bcde	16
Atzeneta	3.9 ± 0.2 a	0.075 ± 0.004 a	52.2 ± 1.5 ab	37.9 ± 2.6 bcdef	19
Font Roja	4.4 ± 0.3 abc	0.081 ± 0.005 ab	55.3 ± 2.0 ab	25.9 ± 1.7 a	18
Sevilla	5.2 ± 0.6 abcd	0.091 ± 0.009 abc	57.0 ± 2.6 ab	42.4 ± 2.4 cdef	15
Alhamilla	4.1 ± 0.3 ab	0.087 ± 0.009 ab	48.6 ± 1.6 ab	32.0 ± 1.2 abc	19
Felix	4.5 ± 0.6 abc	0.088 ± 0.011 ab	51.7 ± 2.1 ab	28.7 ± 2.0 ab	17
Chaouen	6.8 ± 0.5 de	0.141 ± 0.012 cd	49.4 ± 1.6 ab	32.9 ± 1.6 abcd	16
Beni Mellal	5.6 ± 0.3 cde	0.109 ± 0.008 bcd	53.0 ± 2.8 ab	44.3 ± 4.3 def	19
Agadir	8.0 ± 0.6 e	0.186 ± 0.022 d	47.3 ± 3.2 a	50.0 ± 5.7 f	19
P	**<0.0001**	**<0.0001**	**<0.01**	**<0.0001**	

Significant values ($P < 0.05$) are shown in bold.

Table S2. Pearson correlation coefficients between leaf traits and mean fluorescence values in October 2013, February 2013 and late July 2013 for the 12 *Q. ilex* populations

	October 2012			***February 2013***			***late July 2013***		
	LS	**LW**	**SLA**	**LS**	**LW**	**SLA**	**LS**	**LW**	**SLA**
Fo	-0.45	**-0.61**	**0.62**	-0.07	-0.01	-0.01	**-0.55**	**-0.63**	0.24
Fm	**-0.77**	**-0.84**	0.53	-0.49	-0.39	-0.12	**-0.74**	**-0.73**	0.29
Fv/Fm	**-0.62**	-0.52	0.02	-0.56	-0.47	-0.08	-0.45	-0.34	0.03
ΦPSII	0.13	0.20	-0.11	**-0.73**	**-0.66**	0.04	-0.37	-0.35	0.22
Fq'	-0.41	-0.36	0.20	**-0.80**	**-0.73**	0.08	-0.55	-0.53	0.32
qP	0.24	0.29	-0.16	**-0.59**	-0.54	-0.02	0.07	0.01	0.15
NPQ	0.08	0.11	-0.26	**0.78**	**0.75**	-0.25	0.37	0.39	-0.29

Significant regressions ($P < 0.05$) are shown in bold.

Table S3. Pearson correlation coefficients between leaf traits and mean variation (losses or increases) of fluorescence variables measured in February 2013 and late July 2013 compared with October 2012 values for the 12 *Q. ilex* populations

	February 2013			***late July 2013***		
	LS	**LW**	**SLA**	**LS**	**LW**	**SLA**
Fo	0.30	0.48	-0.49	**-0.76**	**-0.75**	0.05
Fm	-0.17	-0.02	-0.38	-0.46	-0.42	0.02
Fv/Fm	-0.46	-0.39	-0.10	-0.15	-0.04	-0.22
ΦPSII	**-0.74**	**-0.70**	0.04	-0.49	-0.50	0.28
Fq'	**-0.75**	**-0.69**	0.01	-0.45	-0.45	0.26
qP	**-0.65**	**-0.63**	0.05	-0.40	-0.37	0.00
NPQ	0.44	0.40	0.05	0.34	0.33	-0.09

Significant regressions ($P < 0.05$) are shown in bold.

Table S4. Pearson correlation coefficients between mean climate variables at each population site and mean fluorescence values in October 2012 (a), February 2013 (b) and late July 2013 (c)

a)	***October 2012***							
	T (ºC)	**Pp (mm)**	**AI (unitless)**	**Tmax (ºC)**	**Tmin (ºC)**	**PET (mm)**	**Pp-PET (mm)**	**Thermal oscillation (ºC)**
Fo	**-0.64**	0.37	0.51	**-0.72**	-0.42	**-0.66**	0.62	-0.35
Fm	**-0.71**	0.14	0.34	-0.42	**-0.72**	**-0.66**	0.49	0.17
Fv/Fm	-0.22	-0.33	-0.21	0.33	-0.54	-0.12	-0.11	**0.75**
ΦPSII	**0.62**	-0.37	-0.51	**0.72**	0.39	**0.64**	**-0.61**	0.39
Fq'	0.04	-0.54	-0.45	0.40	-0.22	0.09	-0.37	0.56
qP	0.55	-0.15	-0.32	0.56	0.43	0.56	-0.43	0.20
NPQ	-0.07	0.32	0.26	-0.03	-0.04	-0.07	0.23	0.00

b)	***February 2013***							
	T (ºC)	**Pp (mm)**	**AI (unitless)**	**Tmax (ºC)**	**Tmin (ºC)**	**PET (mm)**	**Pp-PET (mm)**	**Thermal oscillation (ºC)**
Fo	-0.04	-0.28	-0.16	-0.29	0.09	0.03	-0.18	-0.35
Fm	-0.37	-0.37	-0.18	-0.11	-0.47	-0.34	0.00	0.26
Fv/Fm	-0.36	-0.32	-0.16	0.11	**-0.58**	-0.35	0.03	0.57
ΦPSII	-0.31	-0.10	-0.04	0.17	-0.51	-0.34	0.15	0.57
Fq'	-0.42	-0.20	-0.08	0.10	**-0.62**	-0.42	0.14	**0.59**
qP	-0.21	0.05	0.04	0.12	-0.32	-0.26	0.19	0.37
NPQ	0.30	0.16	0.09	-0.31	0.54	0.29	-0.09	-0.73

c)	***late July 2013***							
	T (ºC)	**Pp (mm)**	**AI (unitless)**	**Tmax (ºC)**	**Tmin (ºC)**	**PET (mm)**	**Pp-PET (mm)**	**Thermal oscillation (ºC)**
Fo	**-0.81**	0.38	0.54	**-0.80**	**-0.59**	**-0.86**	**0.75**	-0.31
Fm	-0.54	-0.12	0.06	-0.08	**-0.60**	-0.52	0.25	0.41
Fv/Fm	-0.01	-0.54	-0.43	0.44	-0.22	0.05	-0.34	0.61
ΦPSII	-0.07	-0.20	-0.16	0.52	-0.32	-0.04	-0.10	**0.76**
Fq'	-0.21	-0.20	-0.11	0.39	-0.47	-0.19	0.00	0.75
qP	-0.18	0.39	0.37	0.07	-0.17	-0.18	0.34	0.20
NPQ	-0.03	0.27	0.27	-0.42	0.22	0.01	0.15	-0.58

for the 12 *Q. ilex* populations

Significant regressions ($P < 0.05$) are shown in bold.

Table S5. Pearson correlation coefficients between mean climate variables at each population site and mean variation (losses or increases) of fluorescence variables measured in February 2013(a) and late July 2013 (b) compared with October 2012 values for the 12 *Q. ilex* populations

a)	***February 2013***							
	T (ºC)	**Pp (mm)**	**AI (unitless)**	**Tmax (ºC)**	**Tmin (ºC)**	**PET (mm)**	**Pp-PET (mm)**	**Thermal oscillation (ºC)**
Fo	0.47	-0.56	-0.55	0.27	0.43	0.54	**-0.66**	-0.07
Fm	-0.07	-0.47	-0.35	0.08	-0.17	-0.06	-0.24	0.21
Fv/Fm	-0.35	-0.27	-0.13	0.04	-0.51	-0.35	0.06	0.45
PSII	-0.51	0.03	0.14	-0.11	**-0.62**	-0.55	0.36	0.38
Fq'	-0.48	-0.05	0.06	-0.03	**-0.62**	-0.50	0.28	0.45
qP	-0.49	0.11	0.20	-0.22	-0.51	-0.54	0.40	0.19
NPQ	0.22	-0.11	-0.10	-0.21	0.37	0.22	-0.20	-0.49

b)	***late July 2013***							
	T (ºC)	**Pp (mm)**	**AI (unitless)**	**Tmax (ºC)**	**Tmin (ºC)**	**PET (mm)**	**Pp-PET (mm)**	**Thermal oscillation (ºC)**
Fo	**-0.74**	0.30	0.45	**-0.67**	-0.57	**-0.81**	**0.67**	-0.20
Fm	-0.33	-0.20	-0.07	0.08	-0.39	-0.33	0.09	0.40
Fv/Fm	0.02	-0.52	-0.42	0.42	-0.15	0.08	-0.35	0.53
PSII	-0.44	-0.06	0.08	0.23	**-0.62**	-0.41	0.22	**0.71**
Fq'	-0.25	-0.09	-0.01	0.35	-0.47	-0.23	0.09	**0.71**
qP	**-0.64**	0.53	**0.64**	-0.44	-0.52	**-0.65**	**0.71**	-0.02
NPQ	0.01	0.06	0.11	-0.41	0.25	0.06	0.00	-0.60

Table S6. Mean leaf chlorophyll and carotenoids concentrations (± SE) of *Q. ilex* measured in seedlings growing in growth chamber conditions from African populations (Agadir, Beni Mellal and Chaouen) and Iberian populations (Sevilla, Salamanca and León).

	African populations	**Iberian populations**	**P**
[Chlorophyll a + b]	2.15 ± 0.07	2.07 ± 0.07	n.s.
[Carotenoids]	3.68 ± 0.19	3.07 ± 0.19	0.025
n	57	57	

Significant differences between groups are indicated when $P < 0.05$.

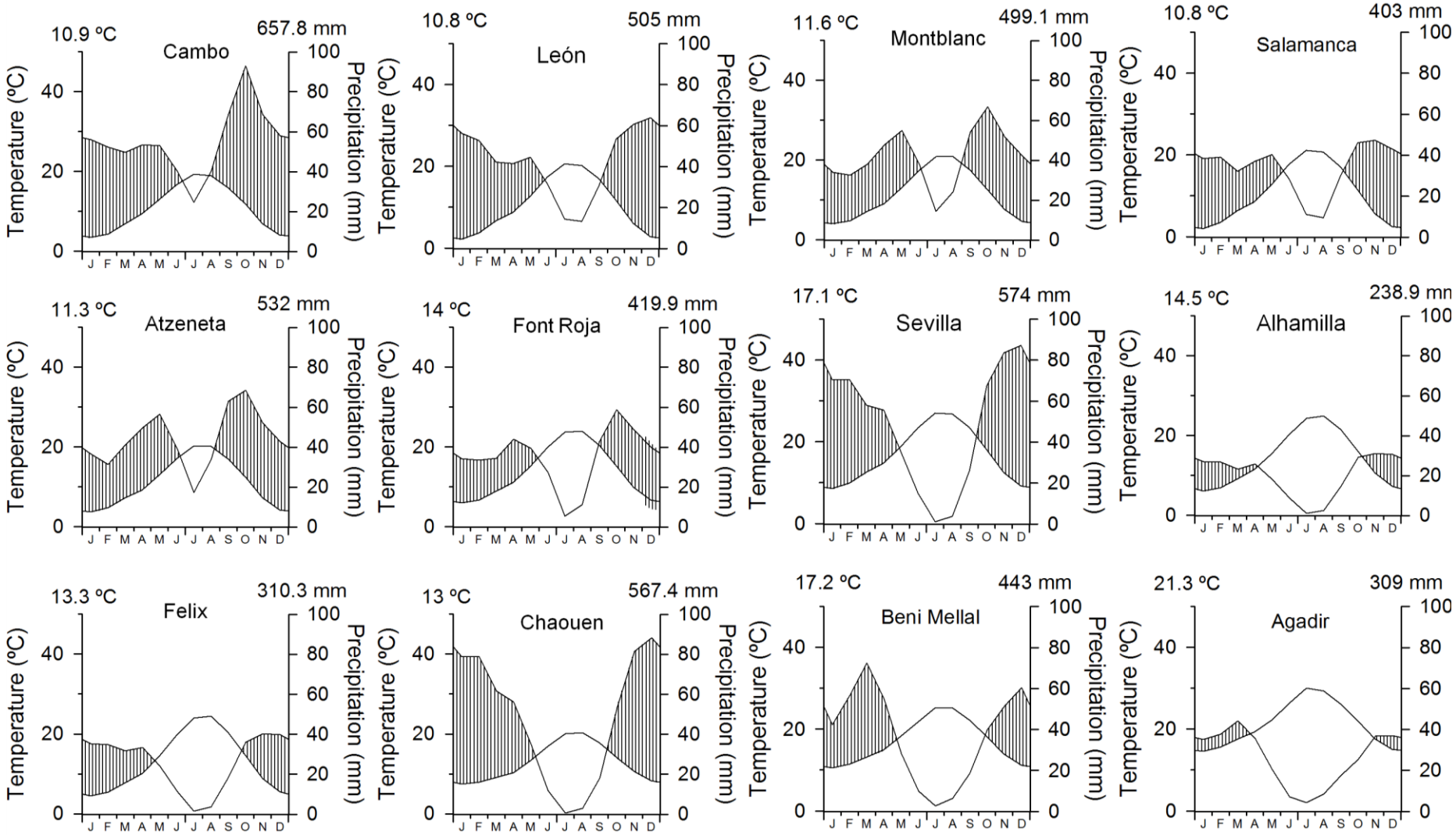

Figure S1. Climodiagrams depicting mean monthly temperature (black line) and rainfall (bars) based on the interpolated E-OBS v10.0 and CRU TS 3.22 climate database and on local climate data for each population studied. Data are the mean for 1950-2015 period.

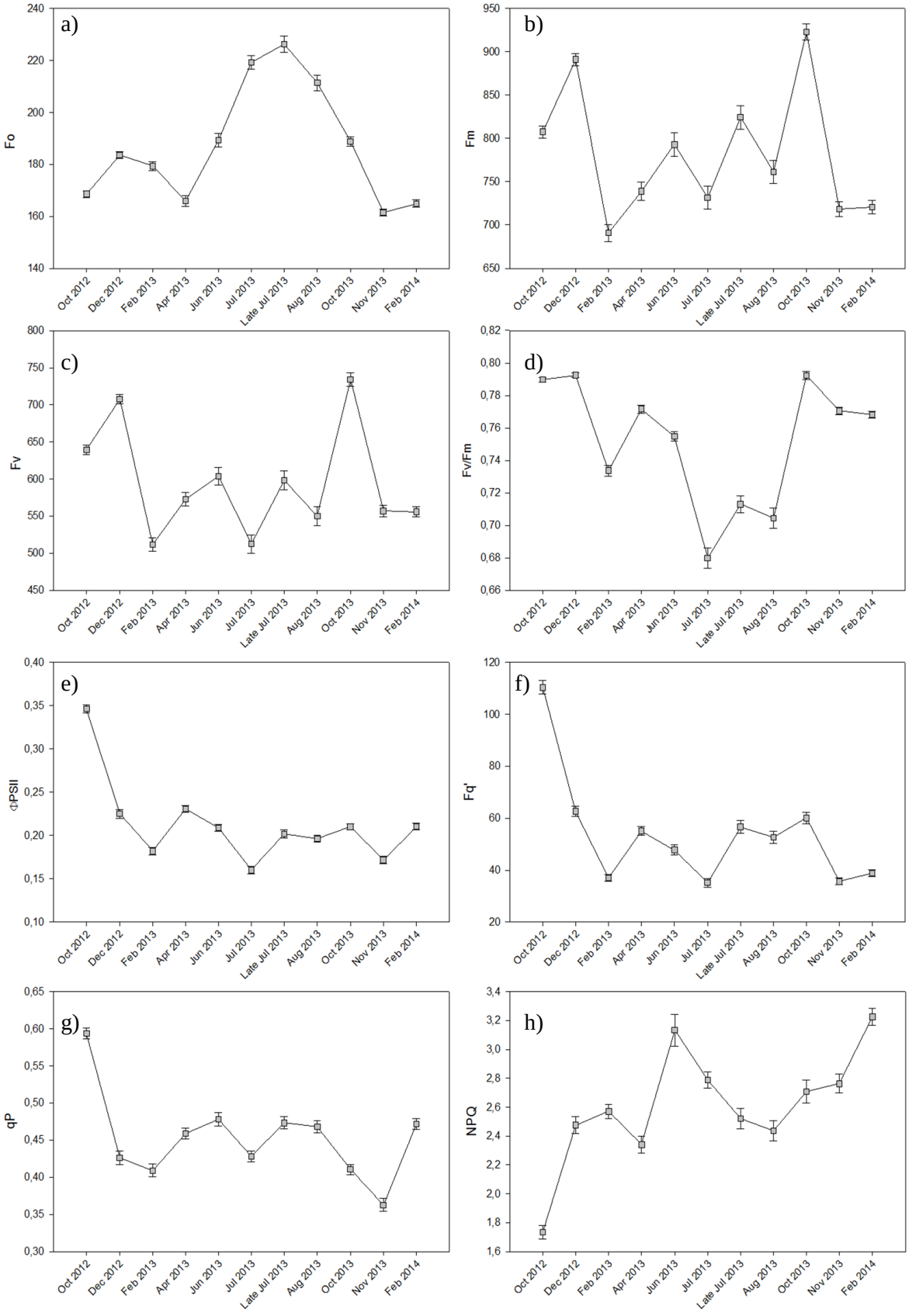

Figure S2. Mean seasonal changes (± SE) in fluorescence emission in both dark and light-adapted leaves for Holm oak taken at midday. Basal fluorescence (Fo) (a), maximum fluorescence (Fm), variable (Fv) (c), maximum photochemical efficiency of PSII (Fv/Fm) (d), actual photochemical efficiency of PSII (Φ_{PSII}) (e), photochemical quenching (Fq') (f), coefficient of photochemical quenching (qP) (g), non-photochemical quenching (NPQ) (h).

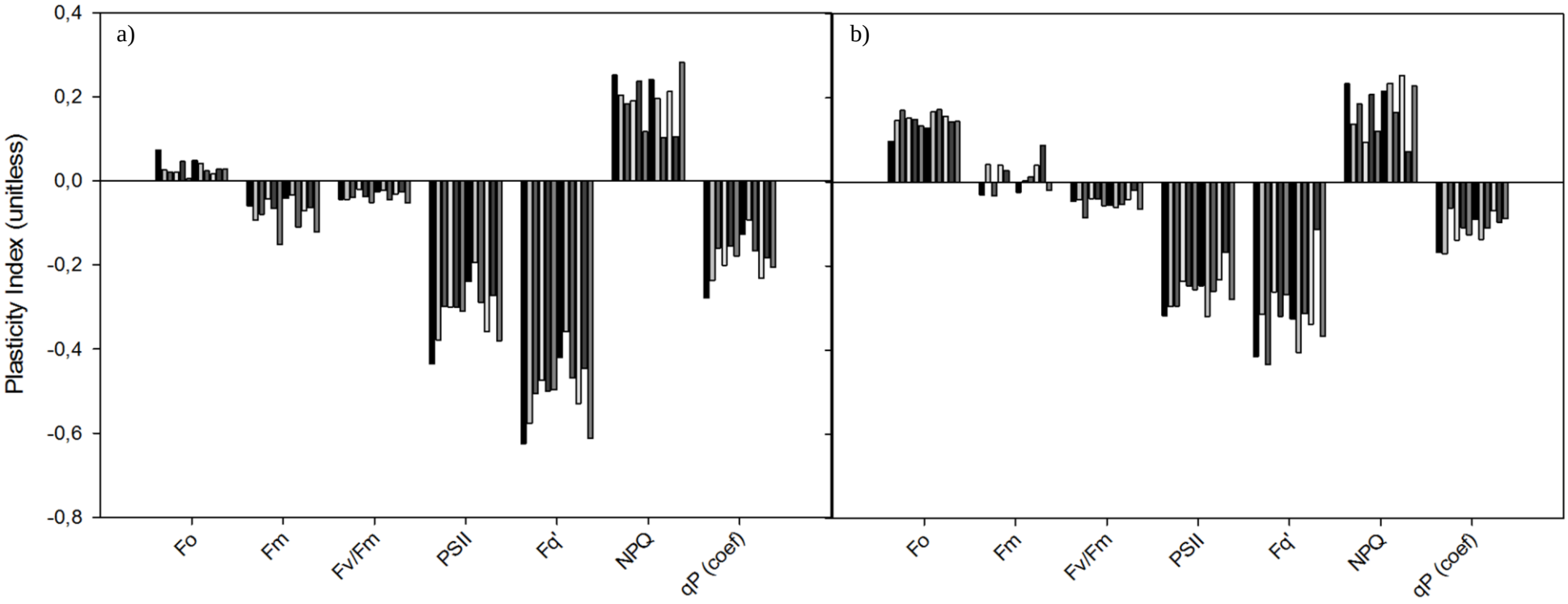

Figure S3. Plasticity index calculated for the main fluorescence variables in the coldest month (February) (a) and in the hottest one (late July) (b). Bars represent populations sorted by latitudinal position (southernmost population at left side and northernmost population at the right)

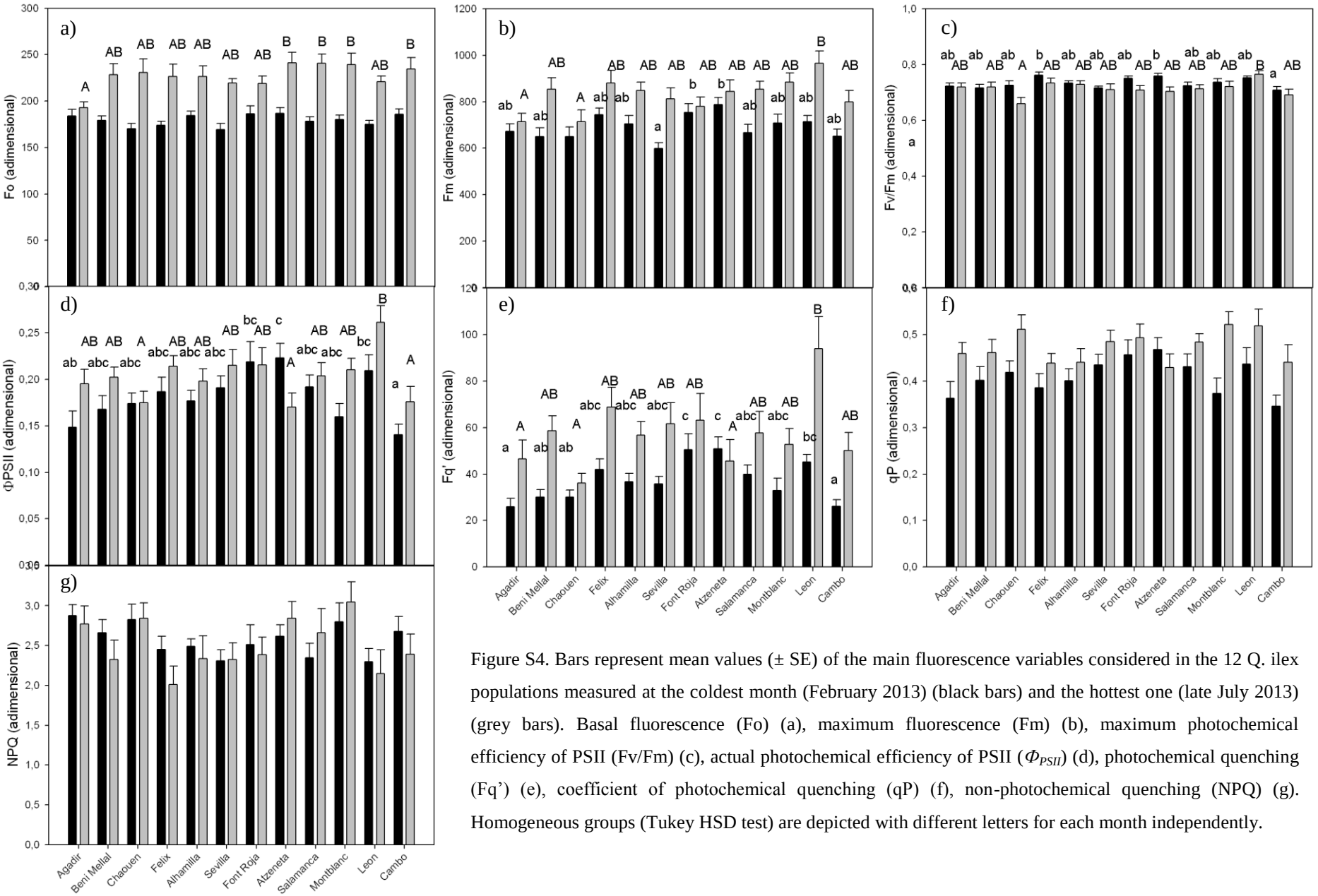

Figure S4. Bars represent mean values (± SE) of the main fluorescence variables considered in the 12 Q. ilex populations measured at the coldest month (February 2013) (black bars) and the hottest one (late July 2013) (grey bars). Basal fluorescence (Fo) (a), maximum fluorescence (Fm) (b), maximum photochemical efficiency of PSII (Fv/Fm) (c), actual photochemical efficiency of PSII (Φ_{PSII}) (d), photochemical quenching (Fq') (e), coefficient of photochemical quenching (qP) (f), non-photochemical quenching (NPQ) (g). Homogeneous groups (Tukey HSD test) are depicted with different letters for each month independently.

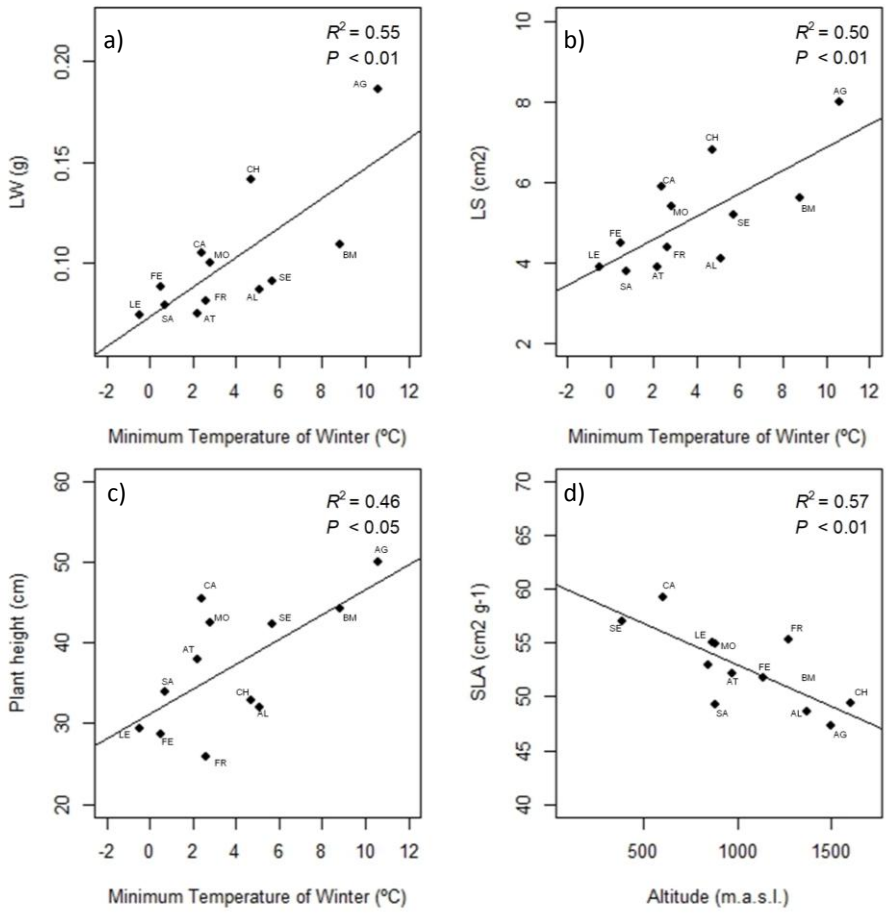

Figure S5. Relationships between both minimum temperature of winter (a, b, c) and altitude (d) in the source of origin and mean morphological traits in seedlings growing under greenhouse conditions from 12 *Q. ilex* populations.

7. DISCUSIÓN GENERAL

El ritmo actual de Cambio Climático justifica el planteamiento de interrogantes en el sentido de si las especies albergarán o no suficiente variabilidad intraespecífica como para poder adaptarse y soportar los futuros escenarios derivados de los modelos climáticos (Alberto *et al.*, 2013). Uno de los objetivos principales del presente estudio ha sido desentrañar la variabilidad intraespecífica y los patrones de variación de una especie de destacada importancia en la Cuenca Mediterránea como es la encina (*Quercus ilex*). El establecimiento de patrones globales de diferenciación de poblaciones en base a su rendimiento y plasticidad a lo largo de gradientes ambientales y de su área de distribución, es clave a la hora de aportar información generalizada para la modelización de la distribución de las especies cuando no se disponen de datos adecuados (Valladares *et al.*, 2014). Los resultados obtenidos contribuyen así a crear un marco de referencia a la hora de formular predicciones sobre los cambios de distribución de las especies en función de su variabilidad intraespecífica.

Globalmente considerados, los resultados del presente estudio ponen de manifiesto la gran variabilidad intraespecífica de la encina en diversos caracteres funcionales a lo largo de su área de distribución. Esta variabilidad emerge probablemente de las complejas interacciones entre la diversidad genética de la especie (Lumaret *et al.*, 2002) y la presión selectiva impuesta por el gran abanico de condiciones ambientales, particularmente climáticas y edáficas, que abarca el área de distribución de la especie (Barbero *et al.*, 1992; Laureano *et al.*, 2008 y 2013).

La variabilidad observada en los resultados proviene, en gran medida, de la diferenciación intrapoblacional; siendo la variabilidad registrada a nivel poblacional menor, pero altamente significativa, a pesar de que los individuos crecían en

condiciones de cultivo homogéneas. Estos resultados sugieren que la diferenciación entre poblaciones puede estar asociada a adaptación local (ver más adelante).

En cuanto a la estructuración espacial de las respuestas fenotípicas de las poblaciones con respecto a su área de distribución, los resultados están de acuerdo –al menos en parte– con la hipótesis de que los individuos de poblaciones nativas de la periferia del área de distribución deben presentar respuestas fenotípicas convergentes en los distintos caracteres morfológicos y fisiológicos considerados. A continuación se detallan los patrones temporales y biogeográficos observados:

Patrones temporales

En el presente estudio setectó un fuerte efecto de la variación entre años en el tamaño de bellota y en la dinámica de germinación de éstas, lo cual es bien explicado por las fluctuaciones climáticas interanuales dentro de cada población (Tabla 4, Capítulo 1). Este resultado revela una fuerte capacidad adaptativa en la especie para hacer frente a la variación en las condiciones ambientales entre años (es decir, refleja su plasticidad fenotípica).

Patrones de borde de área

Las estresantes condiciones ambientales reinantes en los bordes del área de distribución de la encina (bajas temperaturas en el borde norte y sequía y altas temperaturas en el borde sur), pueden explicar las convergencias en las respuestas fenotípicas en ambos bordes. Así, los resultados muestran similitudes en distintos caracteres funcionales en los individuos procedentes de las poblaciones localizadas en la periferia del área de distribución; como por ejemplo, en las características morfológicas de los árboles en sus condiciones naturales, el tamaño de las plántulas, el peso de los frutos y la distribución de la biomasa (Tablas 2, 4 y 5; Capítulo 1). Los resultados pueden considerarse

robustos, ya que estos patrones han sido verificados en distintos años de estudio y en distintas condiciones de crecimiento, a pesar de la fuerte variabilidad interanual observada también dentro de las poblaciones.

Más concretamente, las poblaciones consideradas periféricas mostraron los menores valores de peso de bellota, tamaño de individuos y mayor distribución de biomasa aérea con respecto a la fracción subterránea, así como los árboles más pequeños en sus localidades de origen (Capítulo 1). Varios autores han documentado que el peso de los frutos de *Quercus* tiende a disminuir con la latitud (Koenig *et al*., 2009; Ramírez-Valiente *et al*., 2009). Nuestros resultados coinciden con los de estos estudios, observándose que el tamaño anual medio del fruto se correlacionaba positivamente con la temperatura mínima en invierno del lugar de origen y con la radiación solar de primavera; con lo que, a priori, se esperaría un mayor tamaño de la bellota al descender en latitud. Sin embargo, la población más meridional –la más árida– mostró un tamaño de bellota menor al esperado. Este resultado puede explicarse en base a que en la localidad del extremo sur las condiciones ambientales son tan estresantes que limitan la producción de bellotas viables de mayor tamaño. El hecho de no coincidir con los trabajos previos citados puede deberse a que dichos estudios no incluyeron ambos extremos latitudinales en sus experimentos (ver Aizen & Woodcock, 1992).

En relación a las variables fisiológicas medidas en condiciones de cultivo controlado, cuando las poblaciones fueron cultivadas en condiciones libres de estrés, se observó que las plántulas procedentes de las poblaciones periféricas exhibían comparativamente menor eficiencia fotosintética que las poblaciones núcleo, es decir, las primeras presentaban menores valores de tasa fotosintética y conductancia estomática (*PN* y *GS*), menor fracción de centros de reacción del PSII abiertos (q_P),

menor eficiencia real del PSII (Φ_{PSII}) y menor cantidad de electrones circulando por la cadena de transporte electrónico (F_q') (Figura 1, Capítulo 2). De estos resultados se desprende que las plántulas originarias de los ambientes más estresantes para la especie podrían invertir menos recursos en la operación del aparato fotosintético, en comparación con el resto de poblaciones, evitando así el exceso de especies reactivas de oxígeno (ROS) bajo las condiciones limitantes de los hábitats de los que son originarios; y posiblemente también debido a una mayor inversión en sistemas defensivos y de reparación a expensas de la dotación fotosintética (Takashima *et al*., 2004; Laureano *et al.* 2016), considerándose esta respuesta una estrategia adaptativa. El patrón observado en las variables asociadas a la distribución de la biomasa en los individuos de las poblaciones periféricas (mayor alocación a las estructuras aéreas LAR, S/R) (Figura 4, Capítulo 2), sugiere una estrategia de compensación al bajo rendimiento fotosintético de estas poblaciones. Esta estrategia puede resultar en que, a medio plazo, las poblaciones de la periferia muestren los individuos juveniles con mayor tamaño aéreo observado (Tabla S1, Capítulo 4).

En términos generales, las plántulas de encina mostraron una considerable tolerancia al ozono; ya que, paralelamente a los daños ocasionados por este gas, se observó la activación de un abanico de mecanismos regulatorios, como son el cierre estomático, regulación negativa del PSII, aumento de la disipación de calor en los cloroplastos, etc. Por otra parte, la encina también mostró diferencias en la sensibilidad intraespecífica al ozono (Figuras S2, S3 y S4; Capítulo 3). La menor conductancia estomática característica de las poblaciones nativas de los extremos del área de distribución explica, en parte, su menor reducción de la actividad fotosintética (*PN* y F_v/F_m) que experimentan en respuesta al tratamiento con ozono con respecto al resto de poblaciones (Tabla 3, Capítulo 3); lo que sugiere una estrategia de evitación (Bussotti,

2008). Además, estas poblaciones periféricas mostraron la mayor tolerancia intrínseca al ozono en términos de los principales indicadores del proceso fotoquímico (F_v/F_m, Φ_{PSII}, F_q', q_P, *%D*), manifestando así un patrón convergente (mantenimiento de un mejor rendimiento fotoquímico en condiciones de fumigación) frente a las poblaciones nativas del núcleo (Figura 5, Capítulo 3). Estos resultados apoyan la hipótesis de que los hábitats estresantes favorecen la selección de genotipos mejor adaptados a altos niveles de estrés oxidativo (por ejemplo, frío en el norte del área de distribución, y calor y sequía en el borde sur).

Patrones latitudinales

Paralelamente a los patrones relacionados con la estructura periferia-núcleo del área de distribución, se han detectado patrones asociados a gradientes lineales en el caso de algunos rasgos fenotípicos; por ejemplo, patrones asociados a la latitud de las poblaciones originarias, a la temperatura mínima del hábitat de origen, a su aridez, etc. Concretamente, se ha observado una mayor velocidad de germinación y un mayor porcentaje de germinación cuanto más al sur (Figura 1b,c; Capítulo 1); lo que puede estar relacionado con el tiempo disponible en estas latitudes para que las plántulas puedan alcanzar un tamaño viable antes del inicio de la sequía. Esta estrategia era de esperar como consecuencia de un clima más impredecible e inestable debido al aumento de aridez hacia el sur, requiriéndose de una alta correlación entre la velocidad y porcentaje de germinación con los patrones estocásticos del clima y el acortamiento del período de germinación.

Además, también en las poblaciones del transecto occidental (Capítulo 1) se observó un aumento del tamaño foliar (LS y LW) hacia el sur del área de distribución, tanto en hojas de los individuos adultos en el campo como en las plántulas cultivadas en

condiciones controladas. Sin embargo, cuando se incluyeron poblaciones del transecto oriental (Capítulos 2, 3 y 4), esta tendencia latitudinal en el tamaño foliar cambió, con el resultado de que las poblaciones que presentaban mayor tamaño foliar eran las de los dos extremos del área de distribución (Agadir y Cambo). Todo ello sugiere que, en las poblaciones consideradas de encina, el tamaño de las hojas es un carácter bien conservado tanto en individuos adultos como en plántulas, el cual tiene más que ver con el proceso de colonización de la Península Ibérica por parte de esta especie desde el Norte de África (ver Lumaret *et al.*, 2002; Petit *et al.*, 2005), que con procesos de adaptación local. Parece ser que los individuos marroquíes (caracterizados por hojas grandes) colonizaron la Península Ibérica (Petit *et al.*, 2005); así, los individuos que se iban asentando en la parte oeste tendían a presentar hojas de menor tamaño conforme subían en latitud, quizás debido a la mayor continentalidad de estos hábitats. Por su parte, los individuos que colonizaron el Este de la Península Ibérica tendían a aumentar su tamaño conforme subían en latitud, desde Almería hasta el Languedoc francés, debido posiblemente al aumento de la humedad por su cercanía al Mar Mediterráneo.

Las relaciones observadas entre la cantidad media de electrones que fluyen a través de la cadena de transporte electrónico (F_q') en cada población y su temperatura mínima de primavera, así como con el rango de oscilación térmica registrada en el hábitat de origen (Figura 3a,b; Capítulo 2), indican que las poblaciones procedentes de hábitats más fríos son capaces de dirigir mayor cantidad de energía a través de la cadena de transporte de electrones que las poblaciones de hábitats más cálidos. Este resultado puede deberse, a que las poblaciones de regiones más frías disponen de una estación de crecimiento más corta y la compensan mediante una mayor eficiencia fotosintética (Benowicz *et al.* 2000); lo que sería de esperar también en hábitats cálidos extremos ya que en ellos la estación de crecimiento sería también corta debido a las limitaciones en

la disponibilidad de agua. No obstante, se ha observado que las poblaciones de sitios cálidos muestran un comportamiento opuesto, disminuyendo la cantidad de electrones que circulan por la cadena de transporte electrónico; lo que puede interpretarse como una adaptación a la sequía, ya que la probabilidad de que se produzcan ROS en condiciones de limitación hídrica aumenta con el exceso de flujo de electrones.

Sin embargo, a pesar de que los individuos procedentes de poblaciones con menor temperatura de primavera (y mayor oscilación térmica a lo largo del año) eran los más eficientes en términos de rendimiento fotosintético; éstos no mostraban los mayores tamaños de plántula. Este resultado está en armonía con la relación de compensación entre la baja eficiencia fotosintética de algunas poblaciones y su mayor distribución de biomasa a la fracción aérea (descrita más arriba); así como con otros estudios en los que se ha observado que la longitud del período de crecimiento tiene un mayor impacto en la acumulación de biomasa que la fotosíntesis neta (Oleksyn *et al.* 1998; Benowicz *et al.* 2000). Además, las poblaciones procedentes de hábitats con mayor fluctuación térmica deberían presentar una mayor inversión energética en compuestos celulares que permitan la tolerancia a las temperaturas extremas, lo que resultaría en una menor inversión en crecimiento (Koehler *et al.* 2012; Savage & Cavender-Bares 2013; Laureano *et al.* 2016).

La encina parece muy sensible a la temperatura, como lo demuestra su fuerte respuesta a la baja (febrero) y alta (julio) temperatura estacional; aunque esta respuesta fue muy heterogénea entre poblaciones (Figura 2, Capítulo 4). Las tendencias latitudinales observadas están asociadas con el mayor rendimiento fotoquímico, tanto en invierno como en verano, en las poblaciones procedentes de hábitats más fríos y con mayor oscilación térmica anual. Estos resultados sugieren que los mecanismos involucrados en la tolerancia al frío y al calor están relacionados (e.g. producción de

azúcar intracelular, síntesis de proteínas *shock*) (Larcher, 2000 y 2005; Gimeno *et al.*, 2009; Andivia *et al.*, 2012), y que las poblaciones de hábitats fríos y que registran amplia oscilación térmica han desarrollado mecanismos constitutivos comunes para la protección tanto a las altas como a las bajas temperaturas, es decir, su plasticidad adaptativa es mayor (véase también Mägi *et al.*, 2011); mientras que las poblaciones de sitios cálidos sólo presentan cierta resistencia a la alta temperatura (Figura 4a-d, Capítulo 4). El estudio concreto de la termotolerancia a las altas temperaturas en términos de la fluidez de la membrana de los tilacoides (medido a través de la fluorescencia basal, F_o) mostró una asociación negativa y significativa entre la temperatura crítica de las poblaciones y la aridez en su zona de origen geográfico. Así, la población procedente de la zona más árida del área de distribución de la encina (Agadir) mostró la mayor termotolerancia bajo condiciones de muy alta temperatura, lo que revela una fuerte adaptación local a las condiciones xéricas en esta población del extremo sur.

Otras relaciones asociadas a posibles adaptaciones a la sequía han sido observadas, como es el caso de las ligeras tendencias latitudinales en las que las poblaciones más meridionales exhibían los mayores valores de eficiencia instantánea en el uso del agua (*iWUE*) y –paralelamente– los menores valores de concentración intercelular de CO_2 (*Ci*) (Figura S3, Capítulo 2); sugiriendo que, en sus hábitats de origen (caracterizados por un fuerte déficit hídrico) estas poblaciones han desarrollado un cierre estomático más eficiente. Por su parte, la precipitación anual en el lugar de origen se correlacionó positivamente con el área foliar específica (SLA) media de cada población, indicando que los individuos procedentes de las poblaciones más limitadas por el agua estaban caracterizados por hojas con paredes celulares más gruesas (bajos

valores de SLA); lo que contribuiría a un control más eficiente del intercambio gaseoso, contribuyendo así a limitar la pérdida de agua por transpiración (Bussotti *et al.* 2002).

Consideraciones finales

En los bordes del área de distribución, donde los límites suelen estar impuestos por limitaciones ambientales, el éxito biológico (o *fitness*) de los individuos es generalmente menor que en el centro del área de distribución (Kawecki, 2008). Sin embargo, las poblaciones periféricas no tienen por qué habitar necesariamente hábitats muy desfavorables para la especie (Valladares *et al.*, 2014), como ocurre en la población de Cambo; ya que la encina es capaz de soportar temperaturas inferiores a las registradas en el límite norte. A pesar de ello, esta población es junto con Agadir, la población menos eficiente en términos fisiológicos. Esto sugiere que el área de distribución de *Q. ilex ballota* estaría limitada muy probablemente por la tolerancia a la sequía en el sur; mientras que en el norte puede estar no sólo limitada por los factores ambientales (limitación por las bajas temperaturas a lo largo del año ó subóptimo de temperatura para el crecimiento de la especie), sino también por su baja eficiencia fotosintética. Consecuentemente, esto resultaría en la selección de individuos menos hábiles a la hora de competir satisfactoriamente con otras especies de frondosas en el límite septentrional de la encina, bajo condiciones por debajo del óptimo de temperatura para el crecimiento de ésta, como es, por ejemplo, *Quercus pubescens* (observación personal).

En resumen, del estudio de las distintas poblaciones de encina se desprende que la variabilidad dentro de esta especie recae en gran parte en las poblaciones que habitan los márgenes del área de distribución. Por ello, es de enorme importancia la incorporación de las poblaciones de los bordes del área de distribución a los modelos de

proyecciones futuras que estudian los posibles cambios de distribución geográficos de las especies como consecuencia del Cambio Climático. Este estudio sugiere que las poblaciones periféricas tienen un importante valor para la conservación, ya que éstas pueden ejercer de almacenes a largo plazo para la especie de recursos genéticos, de plasticidad fenotípica, y por todo ello, de capacidad adaptativa.

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8. CONCLUSIONES

1. En condiciones naturales los individuos adultos de encina pertenecientes a las poblaciones de la periferia del área de distribución de *Q.ilex* muestran una menor altura, menor área basal, menor diámetro de copa y menor concentración de nitrógeno foliar que los árboles de las poblaciones del núcleo del área de distribución; todo ello sugiriendo un grado mayor de estrés en las localidades periféricas.

2. Las poblaciones de encina originarias de hábitats localizados en la periferia del área de distribución muestran frutos de menor tamaño y plántulas con una mayor biomasa aérea con respecto a la subterránea (S/R) en comparación con las plántulas originarias de las poblaciones del núcleo del área de distribución.

3. El peso anual de los frutos de encina disminuye con la latitud, posiblemente como consecuencia de la disminución de la temperatura mínima de invierno y de la radiación solar de primavera en las localidades de origen; observándose un mayor tamaño de bellota hacia el sur del área de distribución. La población más meridional (Agadir) presenta frutos de tamaño menor al esperado según su latitud, debido posiblemente a las condiciones ambientales extremas en esta población.

4. La encina presenta gran plasticidad fenotípica dentro de las poblaciones en respuesta a las fluctuaciones climáticas interanuales con respecto al peso de bellota y a la dinámica de germinación.

5. La velocidad de germinación y el porcentaje total de bellotas que germinan aumenta con el tamaño de bellota y con la aridez en la localidad de origen, en lo que parece ser una respuesta a la disminución del intervalo de tiempo disponible

para el crecimiento post-germinación de la plántula al disminuir la latitud; lo que se interpreta como una adaptación que permite a las plántulas alcanzar un tamaño viable antes del comienzo del período de sequía.

6. Las poblaciones de encina muestran diferencias en el rendimiento fotosintético en condiciones de cultivo homogéneas y libres de estrés, siendo las poblaciones periféricas las que presentaron una menor eficiencia en el proceso fotosintético, posiblemente como consecuencia de las fuertes condiciones limitantes en esas localidades. La mayor inversión en la fracción de biomasa aérea en estas poblaciones resultó –a medio plazo– en los individuos de mayor tamaño; lo que se puede interpretar como un mecanismo de compensación.

7. Las poblaciones procedentes de las localidades más frías y con mayor oscilación térmica anual muestran la mayor eficiencia fotoquímica (F_q'), la mayor plasticidad adaptativa a los cambios de temperatura (tanto a frío como a calor, F_v/F_m, Φ_{PSII} y F_q') y los individuos de menor tamaño.

8. Las plántulas de encina consideradas muestran una gran capacidad de adaptación a los cambios estacionales de temperatura, observándose además una considerable diferenciación entre poblaciones en la respuesta a los cambios estacionales de temperatura. La procedencia a hábitats fríos y con mayor oscilación térmica proporciona mayor resistencia tanto a bajas como a altas temperaturas del aparato fotoquímico; en tanto que la procedencia a hábitats cálidos y secos sólo confiere resistencia a las altas temperaturas.

9. Las plántulas de encina consideradas muestran una alta tolerancia al estrés causado por la fumigación con ozono, así como una alta variabilidad intraespecífica en la respuesta a este gas; siendo las poblaciones de la periferia

las más resistentes en términos de eficiencia fotosintética (F_v/F_m, Φ_{PSII}, F_q', q_P, *%D*).

10. Las poblaciones de encina consideradas muestran estrategias de adaptación local a la sequía en algunos caracteres funcionales, como son la mayor termotolerancia en base a la fluidez de la membrana de los tilacoides, la mayor eficiencia en el uso del agua y la mayor área foliar específica en las poblaciones más xéricas del área de distribución.